DIAGNOSIS, EPIDEMIOLOGY AND HUMAN IMMUNE RESPONSE TO CRYPTOSPORIDIOSIS

BY

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SUMMARY

The protozoan parasite *Cryptosporidium* is now widely accepted as a cause of human gastroenteritis. The apparent lack of host specificity and the ability of the organism to undergo its entire life-cycle within the one host has important epidemiological implications. Studies here in Australia and in many other countries have shown *Cryptosporidium* to be an important pathogenic agent in gastroenteritis with an increased incidence in children, a strong rural connection and a possible seasonal trend in some places.

The results of this study show that there are simple and sensitive methods for detecting *Cryptosporidium* which could be incorporated into the standard work up for gastrointestinal disease in the routine laboratory.

The survey found that *Cryptosporidium* was the second most common faecal pathogen found after *Campylobacter jejuni* and therefore the most common intestinal parasite in Tasmania. The disease was found to have definite seasonal trends with peaks in late spring and autumn. Young children were more commonly affected and an association between contact with animals and consumption of unpastuerised milk was shown.

Immunological studies of the different classes of antibodies produced after infection with *Cryptosporidium* show that there is a definite immediate IgA response in most patients followed later by IgM and then IgG. The one AIDS patient with cryptosporidiosis examined in this study showed an almost complete lack of humoral immune response to the infection and one patient possibly became reinfected.

STATEMENT

I, Tiina Hawkesford, declare that this thesis contains no material which has been has been accepted for the award of any other degree in any University or College, and to the best of my knowledge and belief, the thesis contains no material previously published or written by another person except when due reference is made in the text of the thesis.

Signed,

TIINA HAWKESFORD

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1 INTRODUCTION

The investigation of uncomplicated diarrhoeal disease comprises a large proportion of the workload of microbiological laboratories, and routine methods often fail to indicate a causative agent. The list of causative agents, including bacteria, viruses, and parasites continues to increase, (Casemore, Sands and Curry, 1985), and recent interest has focused on the coccidian protozoan, *Cryptosporidium* species. This agent, already well known to veterinarians as a cause of diarrhoea in animals (Angus, 1983), was first recognised in humans in 1976 (Nime et al, 1976). The infection has subsequently been recognised in both immunocompromised subjects (with severe and often life threatening diarrhoea) and normal subjects, predominantly children and young adults, in whom it usually produces a characteristic self-limiting, influenza-like gastroenteritis (Casemore, Sands and Curry, 1985).

Initially it was thought that *Cryptosporidium* was almost entirely zoonotic because of its early veterinary history but it is now known that person to person spread is more common than originally thought (Casemore, Sands and Curry, 1985). The pathogenic mechanism of the organism is still under examination. It has been thought that a mechanical reduction in the mucosal surface and a decrease of many mucosal enzymes may be responsible for lowering the absorptive capacity of the small intestine, producing an

osmotic diarrhoea (Casemore, Sands and Curry, 1985). Also the identification and classification of possible cryptosporidial toxins are the subjects of current investigation (Lefkowitch et al, 1984).

The parasite is notable in its resistance to chemotherapeutic agents, although a wide range have been tested and administered in the more serious cases in humans (Casemore, Sands and Curry, 1985). Recent work with experimental models (Casemore, Sands and Curry, 1985), including induced infection in laboratory animals, cell culture, and fertile hens eggs, as well as microscopical and serological methods for diagnosis, has begun to elucidate the biological and pathogenic mechanisms of the organism. It is now recognised as an important cause of gastrointestinal infection in both normal and immunocompromised subjects throughout the world (Casemore, Sands & Curry, 1985).

Demonstration of an immune response to cryptosporidiosis is essential for assessing pathogenicity, for diagnostic purposes, and for epidemiological studies (Casemore, 1987b). The class of antibodies produced after infection with *Cryptosporidium* is important in the epidemiological study of the organism as well as a useful diagnostic tool in patients with AIDS (Ungar, et al, 1986).

2 LITERATURE REVIEW

2.1 HISTORICAL ASPECTS

The first description of *Cryptosporidium* is credited to Tyzzer in 1907 who found the parasite in the peptic glands of laboratory mice and considered it to be an extracellular species related to the coccidian protozoa (Tyzzer, 1907). He subsequently suggested in 1910, probably incorrectly, that it had already been described in 1884-1985 by J.Jackson Clark, who identified it as a coccidian, *Eimeria falciformii* (Tyzzer, 1912). Tyzzer identified two distinct species - *C. muris* parasitizing the gastric mucosa, and *C. parvum*, infecting the intestinal mucosa (Tyzzer, 1907,1910,1912). Each species appeared to be site as well as host specific. The list of recognised species grew as new animal hosts were identified (Pitlik et al, 1983).

The disease in humans was first recognised in 1976 (Nime et al, 1976) in a 3-year-old female with a severe acute self-limited enterocolitis. The organism was found on rectal biopsy. Until 1981, clinical infections were thought to occur only in immunologically compromised patients, either due to congenital or acquired immune deficiency or as a consequence of treatment with immunosuppressive drugs (Tzipori, 1983a). Subsequently, it has become apparent that the disease is widespread in humans.

2.2 CLASSIFICATION

The causative agent of cryptosporidiosis is a small (2-6um), coccidian protozoan parasite of the genus *Cryptosporidium*, belonging to the family *Cryptosporidiidae*, class *Sporozoasida*, (Levine, 1982). Its systematic position and relationships are given in Figure 1.

The unique characteristic of the genus *Cryptosporidium*, as compared to the other genera of coccidia, is its position and attachment to the microvillar membrane of the hosts' intestinal epithelium. There is some dispute as to whether or not the organism is in fact extracellular (Tzipori, 1983a). Earlier workers suggested that it was never found within the cytoplasm of the cell as in other coccidia (Nime, 1976) and recent transmission electron microscope studies have revealed that the organisms are intracellular (but extracytoplasmic) within parasitophorous vacuoles formed by a continuous covering of microvillus membranes (O'Donoghue, 1984).

Although in the past many species of *Cryptosporidium* have been described (Table 1), and suggestions made that there is only one non host-specific species (Tzipori, 1980b), most have been invalidated because of life cycle or host specificity reasons (Fayer and Ungar, 1986). Recent studies suggest that there are probably only two species

infecting mammals, *Cryptosporidium parvum* and *Cryptosporidium muris* (Fayer and Ungar, 1986). If *Cryptosporidium* is a single species genus, then *Cryptosporidium muris* would be the type species, as this was the first description of this parasite by Tyzzer in 1907 (Casemore, Sands and Curry, 1985).

However, some veterinary workers feel that there may be more recently identified organisms that relate more closely to *Cryptosporidium parvum* (Casemore, Sands and Curry, 1985). There thus remains much controversy over the number of species, with Levine (1984) concluding that there are four existing species (*C. muris*, *C. meleagridis*, *C. crotali*, and *C. nasorum*). More experimental work is required in this aspect before a satisfactory answer will be achieved.

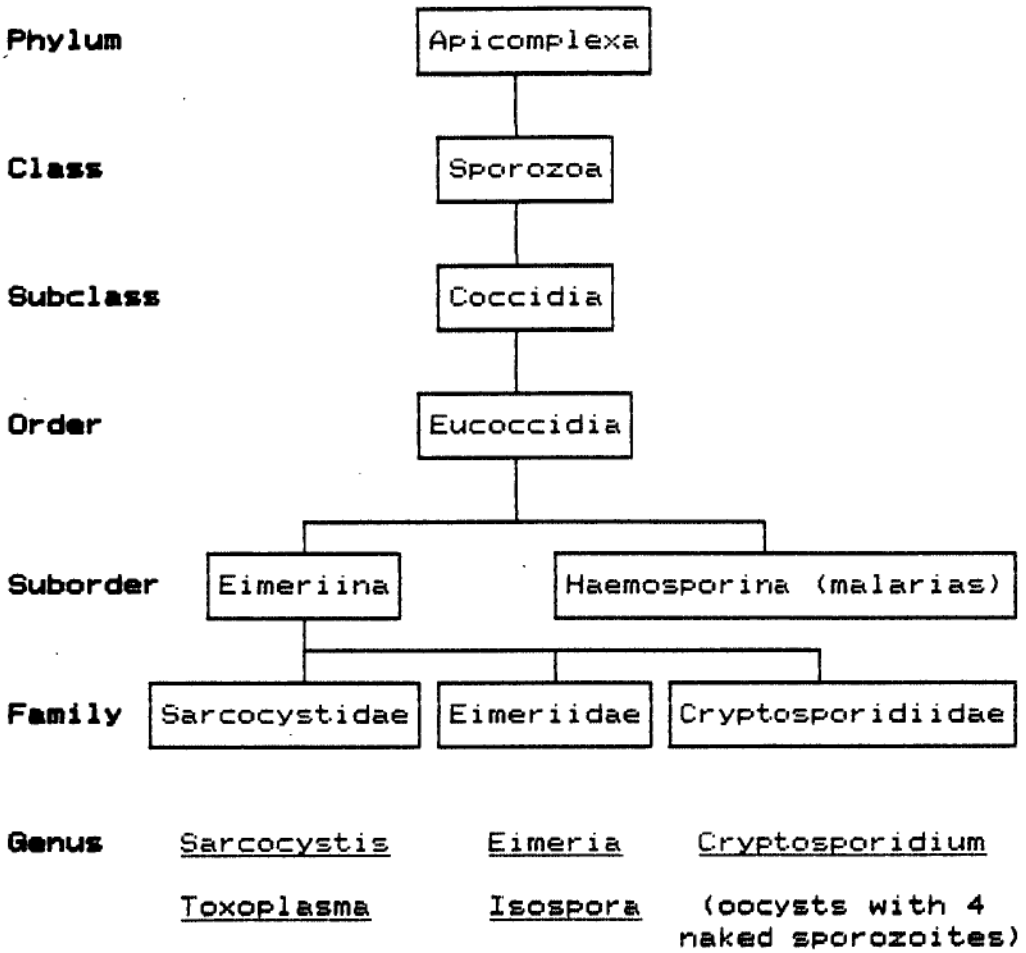


Figure 1. Systematics of Cryptosporidium: simplified scheme, showing relations with other medically important species (Casemore, Sands and Curry, 1985)

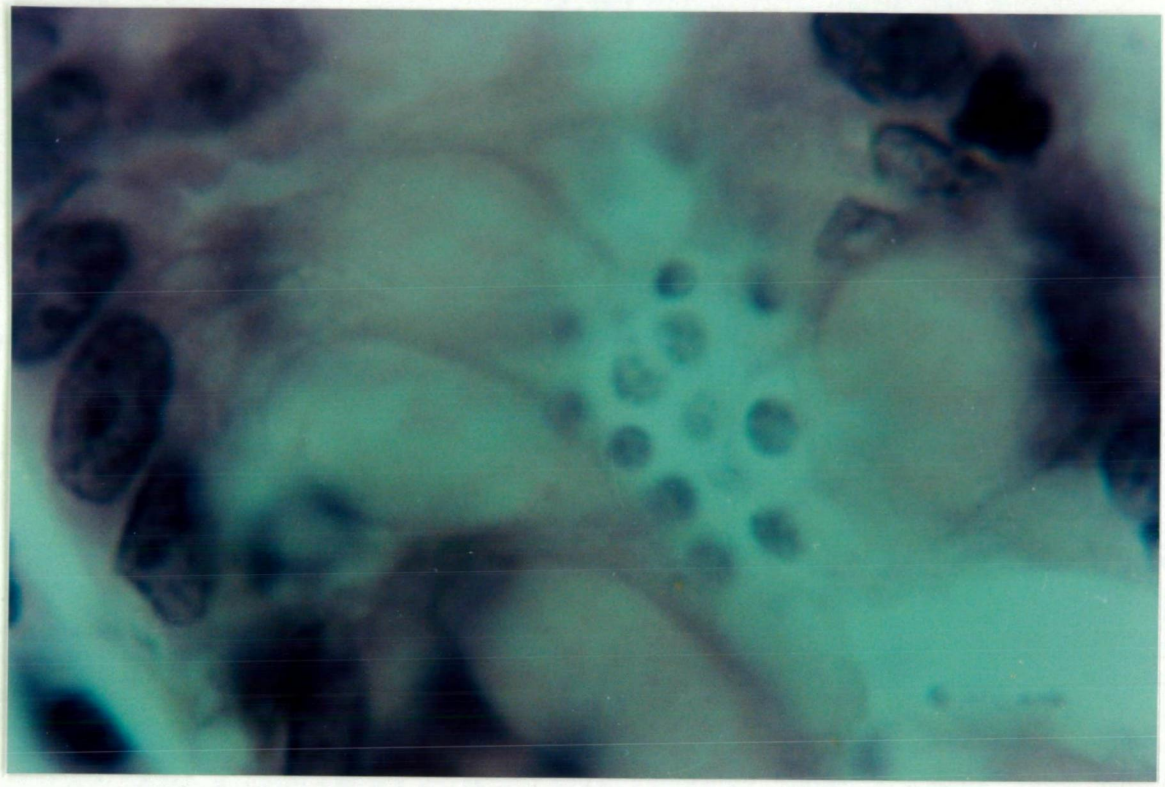


Figure 2. Photomicrograph of *Cryptosporidium* on the gut lining of a calf. H&E stain x1000

SPECIES	HOST
C. agni	*Ovis aries* (domestic sheep)
C. ameivae	*Ameiva ameiva* (lizard)
C. anserinum	*Anser anser* (domestic goose)
C. baileyi	*Gallus gallus* (domestic chicken)
C. bovis	*Bos taurus* (ox)
C. crotali	*Crotalus confluens* (rattlesnake)
C. ctenosauris	Costa Rican lizard
C. cuniculis	*Oryctolagus cuniculis* (domestic rabbit)
C. felis	*Felis catus* (domestic cat)
C. garnhami	Huamns
C. lampropeltis	*Lampropeltis calligaster* (lizard)
C. meleagridis	*Meleagris gallopavo* (turkey)
C. muris	*Mus musculus* domestic mouse
C. nasorum	*Naso literatus* (fish)
C. parvum	*Mus musculus*
C. rhesi	*Macaca mulatta* (rhesus monkey)
C. serpentis	Colubrid,crotalid and boid snakes
C. tyzzeri	*Gallus gallus*
C. vulpis	*Vulpes vulpes* (European fox)
C. wrairi	*Cavia porcellus* (guinea pig)

Table 1. Named species of *Cryptosporidium*. (Meyer, 1988).

2.3 LIFE CYCLE

The life cycle of *Cryptosporidium* is generally similar to that of other enteric coccidia (O'Donoghue, 1984). Viable oocysts ingested by a susceptible host excyst in the gastrointestinal tract releasing the infective sporozoites (Angus, 1983). The sporozoites then undergo asexual proliferation (termed merogony or schizogony) in the lumen and form first-generation meronts each containing 8 merozoites. Each merozoite contains 28 subpellicular microtubules and an apical complex. Merogony is followed by gametogony, in which microgametes fuse with macrogametes to form zygotes with specialised protective outer walls.

Further development to oocysts probably takes place while the organisms are still attached to the enterocytes (Angus, 1983; Bird, 1980). Two types of oocysts are formed, the 'thin-walled' type which remain within the host and are largely responsible for autoinfection, and the 'thick-walled' type which are discharged in the faeces. Both types are sporulated on release from attachment and are therefore infective (Tzipori, 1986). *Caryospora* is the only genus other than *Cryptosporidium* known to sporulate endogenously and to initiate autoinfection (Fayer & Ungar, 1986).

Under experimental conditions, the life cycle may be

as short as 72 hours and this capability of *Cryptosporidium* may have important epidemiological implications (Angus, 1983). Prepatent periods (the interval between infection and oocyst shedding) range from 2 to 7 days in cattle, 3 to 6 days in pigs, 2 to 5 days in lambs, and 5 to 21 days in humans (Fayer &Ungar, 1986). The patent period (duration of shedding) lasted from 1 to 12 days in cattle, 5 to 14 days in pigs, and in immunocompetent humans patency may last for ›30 days (Fayer & Ungar, 1986).

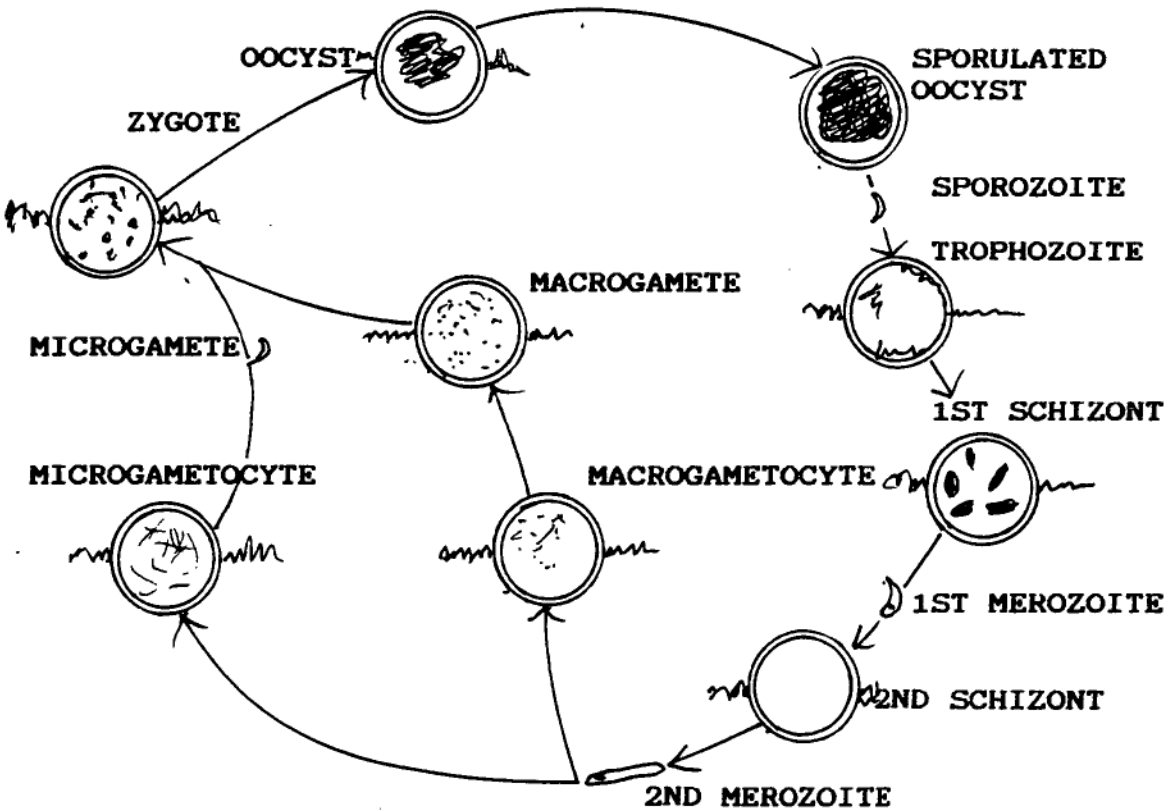


Figure 3. Diagram of the life cycle of *Cryptosporidium* (Tzipori, 1985b).

2.4 EPIDEMIOLOGY

2.41 TRANSMISSION

The ubiquitous nature of the parasite in humans, wildlife (including reptiles and birds), farm livestock, and pets as well as its apparent ability to cross host species barriers implies that the reservoir of infection to humans is large (Casemore, Sands and Curry, 1985). A number of possible and proven cases of person-to-person spread have been documented (Koch et al, 1985; Baxby et al, 1983; Tzipori et al, 1980a; Biggs et al, 1987) including a family outbreak originating from direct animal contact (Ribeiro et al, 1986). Some researchers have suggested that their cases may represent a component of the Travellers' Diarrhoea Syndrome (Jokipii et al, 1983 & 1985a; Holten-Anderson et al, 1983; Soave & Ma, 1985; Ho et al, 1985; Ma et al, 1985; Sterling et al, 1986; Taylor et al, 1988)

Water, raw milk, and foods have all been proposed as sources of infection, although this is difficult to substantiate in the absence of practical concentration techniques or an enrichment culture system (Casemore, Sands and Curry, 1985). Outbreaks of possible waterborne cryptosporidiosis have been reported (Isaac-Renton et al, 1987; Rush et al, 1987; Meyer, 1988). *Cryptosporidium* is known to be widely distributed in surface waters including

raw and filtered drinking water (Meyer,1988). Sewage treatment has been found to be inadequate in removing *Cryptosporidium* from effluent (Madore et al, 1987) and *Cryptosporidium* is known to be resistant to levels of chlorine in excess of those used in water treatment (Casemore, 1987a).

A clear association between bovine cryptosporidiosis and human cryptosporidial infections has been established (Angus, 1983; Holten-Andersen et al, 1983) including the first documented human case who was brought up on a cattle-rearing farm (Nime et al, 1976). The drinking of raw milk or the direct contact with farm animals has been demonstrated as a common cause of cryptosporidiosis (Palmer & Biffin 1987.) The infection has been reported in veterinary students and people working in the live agricultural industry making cryptosporidiosis an occupational hazard (Anderson et al, 1982; Current et al, 1983; Pohjola et al, 1986a). A case of airborne transmission from an infected calf was reported when a veterinary scientist sniffed the air from the gastroscope during treatment of the animal (Hojlyng et al, 1987).

Medical staff caring for patients are also at some risk, as was exemplified when a healthy intensive care nurse acquired cryptosporidiosis from a bone marrow transplant patient with diarrhoea caused by

cryptosporidiosis (Dryjanski et al, 1986). Laboratory staff are also potentially at risk when handling contaminated faecal material due to the organism's resistance to most common laboratory disinfectants, including gluteraldehyde (Weber et al, 1983). A study of the survival of *Cryptosporidium* oocysts in seven laboratory disinfectants showed that only formol saline and ammonia, after 18 hours exposure, were effective in destroying the viability of the oocysts (Campbell et al, 1982). Moist heat treatment of *Cryptosporidium* sp. in calf faeces and intestinal contents caused loss of infectivity after warming from 9°C to 55°C over 15 to 20 minutes or holding at 45°C for 5 to 20 minutes (Anderson, 1985).

2.42 GEOGRAPHIC DISTRIBUTION

There appears to be a worldwide distribution of the organism with reports of human infection from such places as Central Africa (Bogaerts et al, 1984), Southern India (Mathan et al, 1985), Ghana (Addy et al, 1986), United Kingdom (Hart and Baxby, 1985), United States of America (Ratnam et al, 1985; Nime et al, 1976; Wolfson et al, 1985), Guatemala (Cruz et al, 1988), Chile (Weitz et al, 1988), Czechoslovakia (Jirous et al, 1986), Germany (Bertram et al, 1986), Brazil (Weikel et al, 1985), New Zealand (Te Wiata & Lennon, 1985, Carter et al, 1986; Voss & Farmer, 1987) and, of course, Australia (Parker et al, 1985; Palmer, 1986; Tzipori, 1983b; Lumb et al, 1985; Biggs et al 1987) including Tasmania (McColl & Mooney, 1984). The prevalence of cryptosporidiosis worldwide is summarised in Table 2.

Studies on the prevalence of cryptosporidiosis around the world are variable,ranging from 15.4% in Guatemala (Cruz et al, 1988) down to 0.63% in British Columbia, Canada (Montessori and Bischoff, 1985). Baxby et al (1986) found that *Cryptosporidium* was the fourth most common cause of gastroenteritis in a two year prospective survey in a children's hospital.

PLACE	SAMPLES TESTED	% POSITIVE	AUTHORS
AUSTRALIA			
Melbourne	884	4.1	Tzipori 1983
Lismore	140	5.0	Parker et al 1985
Moree	958	1.2	Palmer 1986
Adelaide	9062	0.12	Lumb et al 1985
Adelaide[b]	94	9.6	Lumb et al 1985
Melbourne	2248	2.5	Biggs et al 1987
NEW ZEALAND			
New Plymouth	1273	4.2	Carter 1986
GREAT BRITAIN			
Bristol	867	4.3	Hunt et al 1984
Northallerton	166	7.2	Wyllie 1984
Blackburn	1523	1.6	Wright et al 1984
Liverpool	2573	1.9	Casemore et al 1985
Liverpool[*]	5242	1.4	Hart et al 1985
London[*]	213	3.2	Isaacs et al 1985
England[c]	30043	1.8	Palmer & Biffin 1987
England[d]	20572	0.8	Palmer & Biffin 1987
Newcastle	2197	0.7	Marshall et al 1987
EUROPE			
Finland[*]	154	9.1	Jokipii et al 1983
Finland	5730	2.6	Jokipii et al 1983
France[*]	190	2.1	Arnaud-Battandier 1984
Germany	1160	1.1	Kern et al 1987
Switzerland	2367	1.4	Mai Nguyen 1987
AMERICA			
Massachusetts	2821	2.8	Wolfson et al 1985
Newfoundland	2252	1.2	Ratnam et al 1985
South Carolina	582	4.3	Holley et al 1986
Costa Rica	278	4.3	Mata et al 1983
Guatemala[*]	1280	15.4	Cruz et al 1988
Chile[*]	750	6.4	Weitz et al 1988
AFRICA			
Central Africa	293	7.8	Bogaerts et al 1984
Ghana[*]	474	12.9	Addy et al 1986
Liberia[*]	278	7.9	Hojlyng et al 1984
ASIA			
South India[*]	682	13.1	Mathan et al 1985
Bangladesh	578	4.3	Shahid 1985
Bangkok[*]	410	3.2	Taylor et al 1986

Table 2. Prevalence of *Cryptosporidium* world wide.
[*] children only
[b] aboriginal children only
[c] out patients
[d] in patients
[*] selected patients

2.43 SEASONAL VARIATION

Infection with *Cryptosporidium* is often seasonal, with a higher incidence during the warmer, wetter months (White & Picklo, 1983; Current, 1985; Montessori & Bischoff, 1985; Bossen & Britt, 1985). Variation of from less than 1% prevalence in July to September to 5% in February to April, the Northern Hemisphere summer, has been reported (Baxby and Hart, 1986). Similar reports of summer peaks have been recorded in Canada (Montessori and Bischoff, 1985), and rural Britain (Wyllie, 1984; Hunt et al, 1984). In New Zealand and Australia , spring and early summer have been reported to be the periods of higher incidence (Carter, 1986; Hawkesford, 1987).

Some authors even argue that screening may only be necessary in the summer and autumn months during which time the infection appears to be more prevalent (Tzipori, 1987a). Other authors such as Casemore, Sands and Curry in 1985 and Mathan et al in 1985 found there to be no obvious seasonal variation, which may be related to the climatic conditions of the areas surveyed. A small seasonal peak was noted in spring and a secondary peak noted in early winter in Great Britain, but these cases may reflect rainfall and the many different farming practices and events that can result in widespread dissemination (Casemore, 1987a).

2.44 AGE DISTRIBUTION

Among the immunologically intact, children appear more prone to clinically significant cryptosporidiosis than do adults (Kocoshis, 1986). Most surveys show that young children usually have a higher prevalence of cryptosporidiosis than do adults (Current, 1985; Ratnam et al, 1985; Hart & Baxby, 1985; Tzipori et al, 1983; Soave & Ma et al, 1985; Montessori & Bischoff, 1985; Tzipori,1987b; Cruz et al, 1988). Studies in children's hospitals have also shown that the very young children (less than 12 months old) represent a high percentage (32%) of those infected (Baxby & Hart, 1986).

A survey immunocompetent children in London with acute or chronic diarrhoea showed a 3.2% incidence of cryptosporidiosis (Isaacs et al, 1985). Studies on cryptosporidiosis in children from under-developed countries have shown a very high incidence in the very young i.e. 13.1% in children aged three years or less in India (Mathan et al, 1985) and also in Liberian children (Hojlyng et al, 1984). Children with *Cryptosporidium* positive stools were significantly more malnourished than children in whom *Cryptosporidium* was not detected in a survey in Israel (Sallon et al, 1988).

Studies done on unselected patients, i.e those not

performed in children's hospitals, also usually show a higher prevalence in the young. Mai Nguyen, in Switzerland, (1987) found that 77% of patients with cryptosporidiosis in the survey were children. Palmer & Biffin (1986), in Great Britain, found a 56% incidence in children aged 15 years or less while Casemore (1987a) found a 69% incidence in the same age group as shown in Figure 4. Outbreaks in day-care nurseries are not uncommon (Taylor et al, 1985; Dupont, 1985; McNabb et al, 1985; Alpert et al, 1986) which confirms that person to person spread is common, especially in these situations.

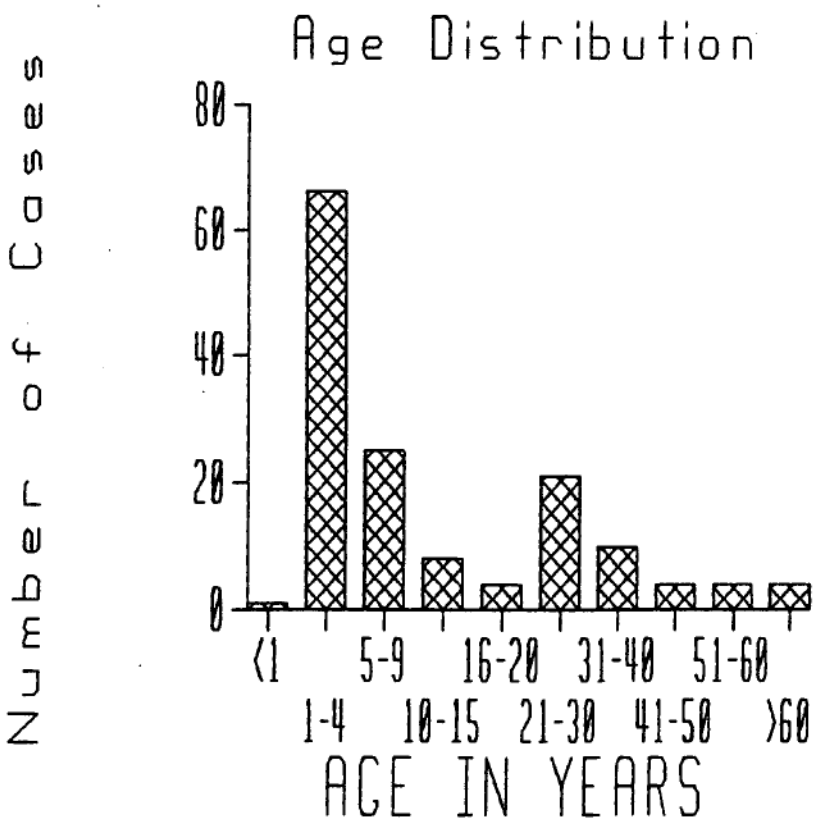


Figure 4. Age distribution of cases (Casemore, 1987a)

2.5 PATHOGENESIS

Animal studies show that the lower small intestine is the organ most severely affected although it can be seen in specimens obtained from the stomach through to the rectum (Pitlik et al, 1983). The most common changes observed are stunted, fused and swollen mucosal villi, coated with immature absorptive cells, while the *lamina propria* is moderately infiltrated with macrophages, neutrophils and eosinophils.

Infection is initiated by the organism forming a stable attachment to the surface of the intestinal mucosa followed by penetration into the epithelial cell (Casemore, Sands and Curry, 1985). Diarrhoea is caused by maldigestion due to loss of membrane-bound digestive enzymes in the upper small intestine and reduced capacity for absorption in the ileum (Tzipori, 1985b). The resulting osmotic diarrhoea coupled with the malabsorption of lactose results in a fermentation process in the lower bowel which may account for the offensive smelling stools which are often reported in cases of cryptosporidial diarrhoea (Casemore, Sands and Curry, 1985). The role of parasite products released by *Cryptosporidium* is yet to be investigated, but it is thought that they may, at least partly, induce hypersensitivity reactions (Tzipori, 1985b).

The ability of the organism to complete its entire life cycle within the one host, and therefore autoinfect, is an important pathogenic mechanism especially in immunocompromised patients (Tzipori, 1985b).

2.6 HOST DIVERSITY

As well as being a natural disease in farm animals, such as cattle, sheep and poultry, cryptosporidial infections in companion animals and exotic pets have also been found (Angus, 1983). The list of new animal hosts has grown to include snakes, chickens, geese, turkeys, guinea pigs, rabbits, pigs, calves, foals, lambs, Indian jungle cats, dingos and monkeys (Pitlik et al, 1983). Some of the more unusual findings by veterinarians in Tasmania studying cryptosporidiosis in animals include respiratory cryptosporidiosis in a 2-week-old peacock chick (Mason and Hartley, 1980) and in the intestinal mucosa of a two-week-old Angora goat kid (Mason et al, 1981). It has also been seen in the conjunctiva of a duck with conjunctivitis and in the Bursa of Fabricius of a fowl (Mason et al,1986 personal communication).

Lack of host specificity was demonstrated when diarrhoeic bovine faeces containing oocysts infected not only calves but also six other species (Angus, 1983). Work has also been carried out with gnotobiotic lambs to prove the enteropathogenicity of *Cryptosporidium* (Snodgrass et al, 1984), and with congenitally athymic (nude) mice to show that T-cells are required for recovery from cryptosporidiosis (Heine et al, 1984).

Sprague-Dawley rats were used in an experiment for finding an effective treatment by immunosuppressing them with hydrocortisone acetate and maintaining them on a low protein diet (Brasseur et al, 1988). The results suggested that this experimental model provided a procedure for screening candidate therapeutic agents (Brasseur et al, 1988).

Hyperimmune serum, raised against oocysts in rabbits, reacted against oocysts in infected cell culture and in the small intestine of piglets, but not against other endogenous stages, and, conversely, hyperimmune serum raised in gnotobiotic piglets which received, after recovery from gut infection, repeated injections of gut scrapings, reacted against other endogenous stages but not against oocysts (Tzipori, 1985b). Experimental studies on organisms found in unusual hosts such as fish or snakes, or from peculiar sites such as the conjunctival sac, trachea, or from the kidney of infected birds, for evidence of biological differences, will no doubt help identify the nature of infectivity of *Cryptosporidium* (Tzipori, 1985b).

The development of a reliable animal model is crucial for the evaluation of various drugs for prophylaxis or therapy (Brasseur et al, 1988).

2.7 EXPERIMENTAL WORK

Cryptosporidium is not an easy organism to study since the oocysts, despite their extreme resistance, gradually lose their infectivity even when stored in preservatives (Tzipori, 1985b). Cultures must be maintained in suitable susceptible laboratory animals, as cell culture has been found to be relatively unsuccessful (Tzipori, 1985b) due to the generation of "thin walled" autoinfective oocysts that are not recovered in the faeces rather than the "thick walled" infective oocysts (Pitlik et al, 1983; Current & Haynes 1984). Experiment has shown 2 to 4 day old laboratory mice to be a useful animal model for the study of many aspects of *Cryptosporidium* infection (Sherwood et al, 1982). Despite continuous efforts, the development of monoclonal antibodies has been largely disappointing (Tzipori, 1985b).

A comparison of the endogenous development of three isolates of *Cryptosporidium* in suckling mice showed that 80% of the oocysts were thick-walled resistant forms that transmitted infection to a new host, and 20% thin-walled autoinfective oocysts thus explaining why immune deficient persons can have a persistent life-threatening cryptosporidiosis in the absence of repeated oral exposure to thick-walled oocysts (Current et al, 1986).

Experimental cryptosporidiosis in newborn calves resulted in the establishment of the organism in the jejunum, ileum, caecum and colon. The middle and lower jejunum and ileum appeared to have the largest number of organisms at 5 to 7 days post inoculation (Anderson, 1984).

An experiment to separate oocysts from calf faeces and then excyst the sporozoites was performed and then the resultant infective (sporozoite) stage exposed to immune bovine serum showing that the sporozoites were completely inactivated (Riggs & Perryman, 1987).

Cryptosporidium oocysts in faeces may be stored for up to 6 months before use in a 2.5% solution of potassium dichromate at 4°C (Campbell et al, 1983). There may be evidence to show that storage of oocysts in potassium dichromate may render any contaminating bacteria non viable whilst retaining the viability and infectivity of the oocysts (Arrowood & Sterling, 1987).

The use of Percoll discontinuous density gradients for separating *Cryptosporidium* from faeces has proved useful in providing a concentrated and largely bacterium-free inoculum for preparing high-titre antisera in rabbits (Waldman et al, 1986). Also the separation of oocysts from faecal debris may be performed using sucrose density grade centrifugation and glass bead columns (Heyman et al, 1986).

The use of both the discontinuous sucrose and isopycnic Percoll gradients has also been advocated (Arrowood & Sterling, 1987), resulting in large numbers of viable contaminant-free *Cryptosporidium* oocysts and sporozoites from faeces.

Experimental *Cryptosporidium* infections in chickens showed that chicks inoculated orally had cryptosporidia in their trachea, whereas chicks inoculated intratracheally had cryptosporidia in their trachea, bronchi, air sacs, in the ducts of the salivary glands and nasal turbinates and had clinical signs of respiratory tract disease. None of the chicks died or had intestinal disease (Lindsay et al, 1986).

Chemoprophylaxis of *Cryptosporidium baileyi* infections was attempted unsuccessfully by feeding 4 groups of chicks diets containing 3 mg of halofuginone/kg of feed, 60 mg of salinomycin/kg, 75 mg of lasalocid/kg, or 110 mg of monensin/kg (Lindsay et al, 1987). This lack of efficacy against *C. baileyi* infections in chickens is similar to that reported for treatment or chemoprophalaxis of *C. parvum* infections in mammals (Lindsay et al, 1987).

2.8 INFECTION IN HUMANS

2.81 SYMPTOMS

In much of the available literature, cryptosporidiosis in humans is described as a short-term, often cholera-like, diarrhoeal illness in immunocompetent persons, or as a prolonged, life-threatening, cholera-like diarrhoeal illness in immune deficient persons (Current et al, 1983). Fluid loss of 10 to 20 litres per day was not uncommon in AIDS patients and the infection was often an important contributing factor in the death of these patients (Berk et al, 1984). In the immunocompetent subjects, the clinical features are an influenza-like illness with watery (and usually foul smelling) diarrhoea, cramps, fever, malaise, and nausea lasting 5 days to 2 weeks (Casemore, Sands and Curry, 1985). The organism may continue to be excreted for up to 2 weeks after the diarrhoea has resolved (Casemore, Sands and Curry, 1985). In contrast, and immunocompetent patient suffered 5 weeks of severe diarrhoea with marked weight loss and renal failure possibly as a consequence of cryptosporidiosis (Edelman & Oldfield, 1988).

The incubation period for the disease can vary from 4 to 12 days (Jokipii et al, 1983 & 1986) and timing of symptoms and oocyst excretion can vary greatly between patients (Jokipii et al, 1986 & 1987). Anorexia and

vomiting are also prominent features of the disease and may in fact precede the onset of diarrhoea (Casemore, 1987c).

Symptom free infections with *Cryptosporidium* in developed countries are not very common as was shown by two studies which both failed to detect oocyst excretion from patients without diarrhoea (Tzipori, 1985a). Conversely, in developing countries the frequency of symptomless carriage is higher, and a study performed in southern India showed that 9.8% of a control group had *Cryptosporidium* oocysts in their stools (Mathan et al, 1985). Also *Cryptosporidium* was sometimes found associated with other pathogens , especially other parasites,in patients from developing countries (Mathan et al, 1985; Bogaerts et al, 1984; Taylor et al, 1986), whereas most studies in developed countries found it to be the only pathogen present (Tzipori, 1985a). The reason for this difference is probably that apparently healthy people in developing areas have a very high intestinal carriage rate of pathogens due to immunity provided by maternal antibodies or exposure in infancy which primes the immune system for active immunity (Albert, 1986).

Recently, *Cryptosporidium* has been described as a cause of laryngotracheitis, when an 8 month old immunocompetent boy from a remote rural village in Papua New Guinea presented with cough, shortness of breath,

fever, and a hoarse cry and cryptosporidial oocysts were found in the tracheal aspirate (Harari et al, 1986).

Perinatal infection with *Cryptosporidium* resulted in failure to thrive and respiratory distress of a baby but no evidence of transplacental transmission, which highlights the potential seriousness of cryptosporidial gastroenteritis in pregnancy (Dale et al, 1987).

An association between *Cryptosporidium* and *Giardia lamblia* has been described in one study where 33% of the patients with cryptosporidiosis also had *Giardia* trophozoites or cysts (Wolfson et al, 1984).Other studies with similar findings of *Cryptosporidium* and *Giardia* such as Palmer, 1986 (40%), Jokipii et al, 1983 (20%) endorse these findings. A study of the clinical features of both giardiasis and cryptosporidiosis found them to be similar (Jokipii et al, 1983). A survey of travellers from Finland to Leningrad in the USSR showed that a large number (38%) of the students concerned contracted either *Cryptosporidium*, *Giardia lamblia* or both after drinking the water (Jokipii et al, 1985b).

SYMPTOMS	CRYPTOSPORIDIOSIS	GIARDIASIS
Diarrhoea	+++	+++
Abdominal pain	+++	+
Vomiting	++	+
Flatulence	++	+++
Fatigue	+++	+++
Anorexia	++	+++

Table 3. Summary of clinical features of cryptosporidiosis compared with those of giardiasis (Jokipii et al, 1983).

2.82 AIDS AND CRYPTOSPORIDIOSIS

Clinical evidence suggests that both major branches of the immune system are required for recovery from cryptosporidiosis (Tzipori, 1985b; Ungar et al, 1986). The T4 helper lymphocyte depletion described in patients with AIDS appears to also involve the small-bowel mucosa where the immunologic reaction to this intramembranous protozoan occurs (Janoff & Reller, 1987). One study of AIDS patients revealed a decrease in the number of IgA-producing plasma cells in intestinal biopsy samples, even though levels of circulatory IgA were normal (Ma, 1987). Therefore, in immunocompromised patients, the disease is severe and often fatal and also the correlation between *Cryptosporidium* and

AIDS has led to the proposal that it should be included as an important factor in the differential diagnosis of the latter condition (Current,1983).

Until the recent emergence of AIDS, cryptosporidiosis had not been well described as an opportunistic pathogen (Cooper et al, 1984). Since then there have been numerous reports of intestinal cryptosporidiosis in patients with AIDS, mainly from the United States of America (Lerner et al, 1984; Whittner et al, 1984). Other reports include a Frenchman who probably acquired AIDS through transfusion with Haitian blood (Andreani et al, 1983), a 13year-old German boy with haemophilia A, lymphocytopenia and antibodies to Human Immunodeficiency Virus (HIV) (Bertram et al, 1986), and an Australian male homosexual (Cooper et al, 1984).

Of a survey of 57 patients with AIDS and cryptosporidiosis, 42 died, and the parasite was rarely eradicated (Casemore, Sands and Curry, 1985). Cases of cryptosporidiosis in patients in Australia with AIDS have been reported (Cooper et al, 1984), and a recent survey in Victoria found seven immunocompromised patients with the disease (Biggs et al, 1987). Four had NHMRC category B or C HIV infection and three had AIDS (Catagory A HIV infection) (Biggs et al, 1987)

AIDS patients with cryptosporidiosis usually present with marked weight loss due to enormous fluid loss (3-20 litres of watery stools per day) and death attributed to the diarrhoea and malnutrition often results (Berkowitz, 1985; Rolston & Fainstein, 1986). Bowel movements may be as frequent as 20-25 per day (Rolston & Fainstein,1986). The diarrhoea is of the secretory type since it persists despite fasting (Guarda et al, 1984).

As well as causing protracted diarrhoea in AIDS patients, the organism has also been found in other parts of the body, including the stomach (Garone et al, 1986) where it was located on the inflamed pyloric ring, causing partial outlet obstruction. Postmortem findings in four patients with AIDS showed the protozoa in their typical location attached to the luminal border of the epithelial gastrointestinal cells (Guarda et al, 1984). Of the four patients, two were diagnosed as having cryptosporidiosis antemortemly, while the two remaining patients were identified at autopsy (Guarda et al, 1984). One of the patients had diffuse infection from the stomach to the rectum and including the gallbladder (Guarda et al, 1984).

A case of cryptosporidial and cytomegaloviral hepatitis and cholecystitis was described in which a patient with AIDS of two years duration with Kaposi's sarcoma was found to have *Cryptosporidium* in a liver biopsy

specimen (Kahn et al, 1987).

Radiologic abnormalities were found in a review of barium studies on the stomach and small intestine in 13/16 AIDS patients with cryptosporidiosis (Berk et al, 1984). These abnormalities included thickened mucosal folds, fragmentation and flocculation of the barium, and narrowing and rigidity of the gastric antrum (Berk et al, 1984).

A study on five patients with AIDS and persistent cryptosporidiosis showed high indirect immunofluorescent titres to *Cryptosporidium* sp., while two subjects with normal T-cell function, hypogammglobulinaemia, and persistent cryptosporidiosis had no detectable titres, suggesting that functional cellular and humoral immunities are necessary to clear infection (Campbell & Current, 1983)

A study involving detection of IgG and IgM antibodies to *Cryptosporidium* in immunocompromised persons, showed a possible lack of IgM response in some patients (Ungar et al, 1986). This may have been due to delay in collection of serum, in which case IgM antibodies may already have disappeared, or the patient may have been incapable of an immune response, or they may have been reinfected and stimulated only a direct IgG antibody recall. Three of the AIDS patients in this study had positive serology before the finding of *Cryptosporidium* oocysts in their stools,

making these assays particularly useful in these populations (Ungar et al, 1986).

A case study of a renal transplant patient with IgA deficiency revealed that the patient died from complications resulting from cryptosporidiosis and may have been the result of the IgA deficiency (Weisburger et al, 1979). Absence of IgA may also have been responsible for the death of a young boy with congenital hypogammaglobulinemia and chronic cryptosporidiosis (Lasser et al, 1979) and a 36 year old man with hemophilia, common variable hypogammaglobulinemia and AIDS (Koch et al, 1983).

Cryptosporidium oocysts were detected during the screening of sputum from an AIDS patients for acid-fast bacilli (Miller et al, 1984) and in the lung on pre and postmortem examination of a bisexual with multiple opportunistic infections (Brady et al 1984). The route by which the infection spread to the respiratory tract from the previously diagnosed intestinal cryptosporidiosis in this 28 year old homosexual male is unknown but possible means include systemic spread or aspiration of infected gastric contents (Miller et al 1984). Pulmonary cryptosporidiosis is usually found associated with other respiratory pathogens such as cytomegalovirus, *Pnuemocystis carinii*, and *Mycobacterium* but *Cryptosporidium* has recently been reported as the sole pathogen in 4 out of 6

AIDS patients with pulmonary cryptosporidiosis (Hojlyng & Jensen, 1988). Sexual practices involving oral-anal contact is undoubtedly the most important mechanism for spreading parasitic infection within the homosexual community (Current, 1983; Phillips et al 1981).

A case of an AIDS patient with intractable diarrhoea due to cryptosporidiosis has been reported in which the patient apparently recovered from the cryptosporidiosis after a course of azidothymidine (AZT)(Chandrasekar, 1987). Although the stool specimens remain negative for cryptosporidia, it is not known if the organism has been eradicated from the patients gastrointestinal tract (Chandrasekar, 1987).

At a London hospital, 26 (11%) patients with AIDS were found to have cryptosporidiosis and 3 of them who had recently been given AZT responded with the cessation of diarrhoea and *Cryptosporidium* were no longer isolated from their stools (Connolly et al, 1988). Most of the patients showed a response to spiramycin and all responded to antidiarrhoeal agents, particularly long acting morphine sulphate (Connolly et al, 1988).

Frequently with AIDS patients, concomitant enteric pathogens are found with *Cryptosporidium* including *Salmonella sp.*, *Shigella sp.*, Enteropathogenic *E coli*,

Yersinia sp, *Campylobacter jejuni*, *Giardia lamblia*, *Trichuris trichiura*, *Isospora belli*, *Ascaris lumbricoides*, *Strongyloides stercoralis*, rotavirus, echovirus, adenovirus, and even *Entamoeba histolytica* and *Mycobacterium avium intracellularis* (Ma, 1987).

A study in Baltimore-Washington of a population of homosexual or bisexual males at high risk for "gay bowel syndrome", asymptomatic carriage of organisms such as *Chlamydia trachomatis*, herpes simplex virus, *Neisseria gonorrhoeae*, and *Giardia lamblia*, mostly in HIV positive men. *Cryptosporidium* was the most common organism found in those men with gastrointestinal symptoms (Laughon et al, 1988).

2.83 LABORATORY DIAGNOSIS

In most reported cases of cryptosporidiosis prior to 1980, the diagnosis had been established by histopathological examination of biopsy or necroscopy specimens or electron microscopic preparations of intestinal mucosa (Current et al, 1985; Lasser et al, 1979). Histopathologically, the cryptosporidial organisms were arranged along the free border of the epithelial cells lining the crypts and the surface of the gastrointestinal tract (Lefkowitch et al, 1984). Oocysts in faeces were first detected and reported by Pohlenz et al (1978) who

worked on calf material stained by Giemsa, and opened the way for diagnosis by non-invasive means.

In the early 1980s acid-fast staining using a cold Ziehl-Neelsen technique was introduced (Henrikson and Pohlenz, 1981). This was followed by the use of other acid-fast techniques including the modified acid-fast and Kinyoun acid-fast methods (Garcia et al, 1983a). A rapid modified Ziehl-Neelsen technique was developed which, although some cysts were left unstained, decreased the risk of false positive results due to staining of yeasts and other acid-fast bodies (Casemore et al, 1984).

Another extremely useful technique is that of Heine (1982), in which equal quantities of faeces and Kinyoun carbol-fuchsin are mixed on a slide and smeared thinly, air dried, and examined using immersion oil and a cover slip. Everything stains pink except the oocysts which are bright and refractile. Because the oocysts contain water and collapse on drying it is necessary to examine the slide within 10 to 15 minutes (Current, 1983).

Later a simple method using safranin and heating was developed (Baxby & Blundell, 1983) and some workers reported it to more sensitive than the earlier techniques (Baxby & Blundell, 1983; Bogaerts et al, 1984).

The use of the fluorescent dye, auramine, instead of

the cold Ziehl-Neelsen provided a rapid and sensitive method for screening large areas of stained material (Payne et al, 1983). This method has since been modified to an even more rapid two step procedure with counter-staining in strong carbol-fuchsin (Casemore, Armstrong and Sands, 1985). The auramine showed a greater affinity for the oocysts than carbol-fuchsin when 3% acid-alcohol failed to decolourise the auramine stained oocysts but decolourised the carbol-fuchsin ones (Nichols et al, 1984).

Combined with concentration techniques such as the new disposable plastic tube device (FPC), and Sheather's sugar flotation a superior method of recovering the oocysts in stools was established (Zeirdt, 1984) and still used extensively (Navin & Juranek, 1984) although some workers believe that concentration is unnecessary due to the fact that numerous oocysts are passed in the acute phase of the illness (Weber et al, 1983). Concentration plus staining using the modified cold Kinyuon acid-fast method was found to be useful during a large outbreak of cryptosporidiosis in two day care centres (McNabb et al, 1985).

Immunofluorescence detection of *Cryptosporidium* oocysts has been tried using an indirect immunofluorescent antibody (IFA) procedure with rabbit antisera and was found to be more sensitive than auramine O in the detection of small numbers of oocysts (Stibbs & Ongerth, 1986). This was

also found to be true with a direct immunofluorescent antibody test (Sterling & Arrowood, 1986) and an indirect immunofluorescent antibody test (McLauchlin et al, 1987) both using monoclonal antibodies conjugated with fluoroscein isothiocyanate (FITC).

An even more sensitive method for detection of *Cryptosporidium* oocysts using a fluorescent monoclonal antibody technique has recently been developed (Garcia et al, 1987) called Merifluor™ and is now available commercially in North America (Meridan Diagnostics, Ohio). Also, a rapid immunoassay using a latex agglutination test showed equal sensitivity to that of direct examination although further study on this method is proceeding (Pohjola et al, 1986b).

It has been suggested that it may be advantageous to use more than one technique for optimal recovery and identification of the organism (Garcia et al,1983b). Undoubtedly, with most of the techniques, experience is necessary to differentiate oocysts from other like-staining bodies and thus the size, shape and staining properties are important for distinguishing between *Cryptosporidium* oocysts, fat droplets and yeasts (Baxby, Blundell and Hart, 1984a).

A comparison of three different staining methods found

the dimethyl sulfoxide modified acid fast stain to be superior in sensitivity to the modified Ziehl-Neelsen and Kinyoun's acid fast stains (Gardner et al, 1985). A more recent study has shown the fluorescent auramine-phenol technique to be more sensitive and easier to perform than other methods including carbol-fuchsin acid fast, safranin, or Geimsa staining (Hawkesford, 1986, personal communication).

Atypical oocysts that do not stain by usual methods have been reported (Baxby, Blundell and Hart, 1987; Collignon, 1987). Such oocysts may only be readily detected in fresh sucrose-phenol preparations and highlights the need for direct examination of all faecal samples (Baxby, Blundell and Hart, 1987)

Other methods that have been developed include the dimethyl sulphoxide modification of the acid fast technique used to monitor cryptosporidiosis in nonhuman primates (Bronsden, 1984). Electron microscopy with negative staining (Baxby et al, 1984b; Tzipori, 1985b) has also been used. The Nomarski differential contrast microscopy may be a useful method for identifying *Cryptosporidium* in a wet preparation (Goldsmid et al 1986).

There has been some suggestion that it may not always be necessary to screen stool specimens for *Cryptosporidium*,

and that seasonality and the type of patient (i.e. immunocompromised or young) should be a factor in deciding the economics of routine investigation (Bissenden, 1986).

2.84 IMMUNE RESPONSE

Even though *Cryptosporidium* develops in a 'pseudeoexternal' location, an immune response has been previously shown (Casemore, 1987b). Mechanisms other than invasion of the mucosa that might provoke an immune response include persorption, which has been shown in candidaemia, and apoptosis, or active cellular self-destruction known to take place in enterocytes and resulting in antigen being presented to the immune system (Casemore, 1987b).

The prevalence of antibodies in 10 animal species, including humans, was found to be 80% in a study using indirect immunofluorescence of infected lamb gut tissue (Tzipori and Campbell, 1981).

An indirect immunofluorescent technique was also used to demonstrate the antibody response to *Cryptosporidium* in humans (Campbell and Current, 1983). Portions of gut ileum from infected 3 day old mice were sectioned and fluoroscein isothiocyanate (FITC)-conjugated antihuman polyvalent immunoglobulin used to detect antibodies in groups of

subjects ranging from those known to be infected, including some with immunodeficiency, to subjects not known to have been exposed to *Cryptosporidium* (Campbell & Current, 1983). Cross-reactivity studies to other intestinal parasites including *Toxoplasma gondii*, *Isospora suis*, and *Sarcocystis bovicanis* showed no significant cross-reactions in the sera tested (Campbell & Current, 1983). Infection with *Cryptosporidium* sp. may not result in protective immunity, since one subject in the study experienced three episodes of cryptosporidiosis in a 1-year period (Campbell & Current, 1983).

Purified *Cryptosporidium* oocysts were used to demonstrate human immune response using an indirect immunofluorescent test (Casemore, 1987b), The different classes of antibodies produced was examined in a controlled study and evidence was found of a poor primary IgG response, with an expected rise and fall of IgA and IgM. (Casemore, 1987b). A marked IgE response in some cases, in line with a rise with a total serum IgE, although typical of infections with nematodes and helminths, was a surprising finding in this study. Results of the study by Casemore (1987b) on paired sera from patients diagnosed as having had cryptosporidiosis are summarised in Table 4.

Case No.	Age (years)	Days after onset	Titres IgG	IgA	IgM	IgE
1	37	9	5	5	5	20
		91	80	20	20	160
2	36	11	10	5	5	20
		112	40	80	30	160
3	30	5	5	5	10	20
		70	5	20	80	80
4	8	5	5	5	10	5
		28	20	40	80	80
5	7	10	5	5	5	5
		21	40	20	20	20
6	30	7	5	5	15	5
		35	5	60	30	5
7	4	14	5	5	5	5
		30	10	160	10	40
8	15	14	5	15	10	10
		24	15	40	160	40
9	30	150	5	160	20	20
		240	5	160	20	20
10	15	98	5	10	20	20
		240	5	5	20	20

Table 4. Paired serum samples from confirmed cases (Casemore, 1987b).

An ELISA technique for the detection of immunoglobulin M and G to *Cryptosporidium* showed a high prevalence of infection in immunodeficient and immunocompetent persons (Ungar et al, 1986). Microtitre plates were coated with oocysts purified by a saturated sodium chloride (NaC) technique and sonicated then to disrupt them. After incubation with the test sera, the plates were washed and either alkaline phosphotase-conjugated goat antibody to human IgG or IgM was added to each well and after further incubation and washing, substrate solution (*p*-nitrophenyl phosphate in diethanolamine) was added and the absorbance read on a microplate colorimeter) (Ungar et al, 1986).

Antibodies were detected in all patients 2 weeks after the illness, with IgM antibodies appearing early in the illness and then disappearing while the IgG antibodies rose in the first 6 weeks and often remained present for 12 months or more (Ungar et al, 1986). There was no evidence of cross-reactivity with other intestinal or protozoan parasites seen with the ELISA (Ungar et al, 1986).

A study to determine which *Cryptosporidium* antigens invoke antibody responses using polyacrylamide gel electrophoresis and laser densitometry found a 23,000 dalton antigen common in 93% of patients studied (Ungar & Nash, 1986).

2.85 TREATMENT

Over 40 antimicrobial agents, including coccidiostats and other antiprotozoan compounds, broad spectrum antibiotics and even anthelmintics have been tested against *Cryptosporidium* without success (Tzipori, 1983a).

The only antimicrobial agent with any demonstrable success against *Cryptosporidium* is spiramycin, a compound also used in the treatment of toxoplasmosis (James and Gillies, 1985). It is a macrolide antibiotic similar to erythromycin, and its use has resulted in symptomatic improvement in 50% of patients with AIDS (Berkowitz & Seidel, 1985). In an uncontrolled study of nine AIDS patients and one bone marrow transplant patient with cryptosporidiosis, all of the infections eventually resolved after treatment with spiramycin (Portnoy et al, 1984).

Recent studies with the drug Arprinocid, which is effective against *Eimeria*, showed that it had a parasitistatic, rather than a parasiticidal effect on *Cryptosporidium* when administered therapeutically in hamster neonates, although further studies may show it to be useful in higher doses (Kim, 1987). The long-acting somatostatin analog, octroetide, was used recently with some degree of success to treat a 26 year-old haemophiliac

with AIDS and cryptosporidiosis (Katz et al, 1988).

DRUGS USED TO TREAT HUMAN CRYPTOSPORIDIOSIS

Amphotericin B	Loperamide	Sulfamethoxazole
Ampicillin	Mepacrine	Sulfadiazine
Carbenicillin	Metronidazole	Sulfisoxazole
Chloroquine	Oxytetracycline	Sulfthalidine
Cholestyramine	Pentemidine	Thiabendazole
Cloxacillin	Penicillin	
Colistin	Piperazine	
Erythromycin	Pyrimethamine	
Gentamicin	Primaquine	
Levamisol	Septrin	

Table 5. List of antimicrobial agents reported to be ineffective against *Cryptosporidium* infection (Tzipori, 1983a).

In a recent case in Australia of chronic diarrhoea in a 3 year old boy with congenital hypogammaglobulinaemia, from whom *Cryptosporidium* was isolated, treatment with hyperimmune colostrum from a cow previously immunized with *Cryptosporidium* antigens was started. Clinical improvements resulted and 8 days after commencement of treatment, no oocysts could be detected in his faeces, suggesting that

the disease course was altered by this unusual treatment (Gilbert, 1986a; Tzipori et al, 1986). Since then this child has developed colonisation of the biliary tree with *Cryptosporidium* with shedding into the bowel. It has been possible to clear the organism from the childs bowel but the gall bladder remains colonised (Gilbert, 1986b).

Treatment of 2 other immunocompromised patients using hyperimmune cow colostrum has also been successful (Tzipori, 1987c). One patient was a 40 year old man with AIDS and the other a 4 year old girl on immunosuppresive therapy for acute lymphoblastic leukaemia and represents both T and B cell abnormalities (Tzipori, 1987c). Hyperimmune bovine colostrum (HBV) can be freeze dried and gamma irradiated without loss of specific antibody, however the therapuetic value of HBC requires assessment in properly conducted clinical trials (Tzipori, 1987c).

A ten year old haemophiliac boy with AIDS and cryptosporidiosis failed to respond to treatment with hyperimmune cows milk and died of unremitting diarrhoea (Spence, 1988 personal communication). Conversely, a child with rhabdomyosarcoma on constant chemotherapy suffered 4 weeks of diarrhoea due to *Cryptosporidium* and despite non-withdrawal of the immunosuppresive treatment the diarrhoea resolved (Oh et al, 1984). A young girl with acute lymphocytic leukaemia and CNS involvement developed

acute diarrhoea due to *Cryptosporidium* and the diarrhoea only resolved after withdrawal of the immunosuppresive treatment (Miller et al, 1983)

The use of bovine colostrum anti-cryptosporidial antibody failed to alter the course of human cryptosporidiosis in two patients with AIDS and one with congenital dysgammaglobulinaemia even though the colostrum administered was shown to contain antibody activity against oocysts and sporozoites of *Cryptosporidium* (Saxon & Weinstein, 1987). Oral bovine transfer factor has been used with some degree of success to treat AIDS patients and risk group patients with cryptosporidiosis (Louie et al, 1987). Of the 8 patients studied, 5 exhibited an improvement but mostly relapsed or expired from other causes and only one patient eventually recovered (Louie et al, 1987)

2.9 FUTURE TRENDS

Further experimental work is in progress to attempt to explain the peculiar epidemiological and biological characteristics of *Cryptosporidium* (Tzipori, 1985b). The question of identification of different species in the community and which of these are pathogenic to man remains to be answered (Meyer, 1988; Katz et al, 1987). Methods for treating water to prevent the waterborne spread of these organisms are needed (Meyer, 1988). In view of the increasing prevalence of AIDS, the development of accurate, rapid techniques of diagnosing cryptosporidiosis and especially for routine laboratories is important. Also, the lack of suitable chemotherapeutic substances effective against *Cryptosporidium* makes it essential that work to find a suitable agent should continue (Casemore, Sands and Curry, 1985) while the development of a suitable vaccine to protect both humans and animals would be desirable (Meyer, 1988).

Cases of cryptosporidiosis in the community should be identified in order to contain human transmission and avoid the spread from immunocompetent to immunodeficient individuals (Kocoshis, 1986). The incidence of cryptosporidiosis in immunologically normal subjects requires further epidemiological and environmental studies (Casemore, Sands and Curry, 1985). Analysis of the

interplay between subpopulations of T and B lymphocytes, antibodies of various classes (such as IgG, IgA, IgM, IgE), and accessory macrophages, eosinophils, neutrophils and mast cells in host-parasite relationships will provide data of interest to the immunologist and pathologist (Mitchell, 1981). Because of its relatively recent intrusion into the realms of medical importance, *Cryptosporidium* is an exciting area for the researcher of the future. It was with these needs in mind that the present study to define the problem of cryptosporidiosis in Tasmania was undertaken. Tasmania is an ideal situation for such studies because of its relative isolation from the rest of Australia and until this study was undertaken the only record of *Cryptosporidium* in humans on the island was the case report by McColl and Mooney (1984).

Real proof that the organism existed Tasmania wide and probably was the cause of significant numbers of cases of gastrointestinal disease could only come from finding the parasite, showing that it produced an immune response in humans and demonstrating the presence of antibodies to *Cryptosporidium* in the different population areas. As little such work had previously been undertaken elsewhere in Australia, or for that matter the world, it was necessary to put together a programme of parasite detection, surveillance and immunological study to prove the hypothesis.

3 COMPARISON OF STAINING METHODS

Many different stains and modifications of stains for detecting *Cryptosporidium* oocysts in faeces have been reported in the literature including the following: Geimsa, acridine orange, rhodamine-auramine, auramine, Kinyoun's, Ziehl-Neelsen, modified Ziehl-Neelsen, safranin, dimethyl-sulfoxide (DMSO) modified and carbol-fuchsin negative stain. Much discussion has prevailed as to the most suitable technique for use in the routine laboratory.

This study will evaluate six of the more widely used techniques to find a simple yet sensitive method that may easily be incorporated into the routine investigation of gastrointestinal disease. Each technique will be assessed by organism quantitation, organism morphology, ease of visualisation and ease of performnace. Based on these comparitive studies, a technique suitable for use in the surveillance of stools for the presence of *Cryptosporidium* oocysts will be instituted.

3.1 MATERIALS AND METHODS

Faecal samples from patients with cryptosporidiosis and infected kid goat material were pooled and used to prepare multiple smears for staining. These smears were then stained following the six different procedures listed in Table 6. Methods used are detailed in Appendix 7.1.

TECHNIQUE	REFERENCE
Carbol fuchsin negative stain	Heine, 1982
Auramine / carbol fuchsin	Casemore, Armstrong and Sands, 1985
Safranin / methylene blue	Baxby, Blundell and Hart, 1984
Giemsa stain	Blakey, 1984
Modified acid-fast (hot)	Garcia et al, 1983
Kinyoun acid-fast (cold)	Ma & Soave, 1983

Table 6. Staining techniques selected for use in the study.

3.2 RESULTS

The stains tested are graded 1 (poor) to 5 (excellent) as to their comparable features in Table 7. The photomicrographs (Figures 5-10) show that all techniques succeeded in detecting the oocysts although some of the techniques stained fewer oocysts and some results were much clearer than others.

Technique	Ease of method	Ease of vision
Carbol fuchsin negative stain	5	3
Auramine carbol fuchsin stain	4	5
Safranin-methylene blue stain	2	2
Giemsa stain	2	1
Modified acid-fast stain	3	4
Kinyoun stain	3	4

Table 7. Summary of the evaluation of the stains.

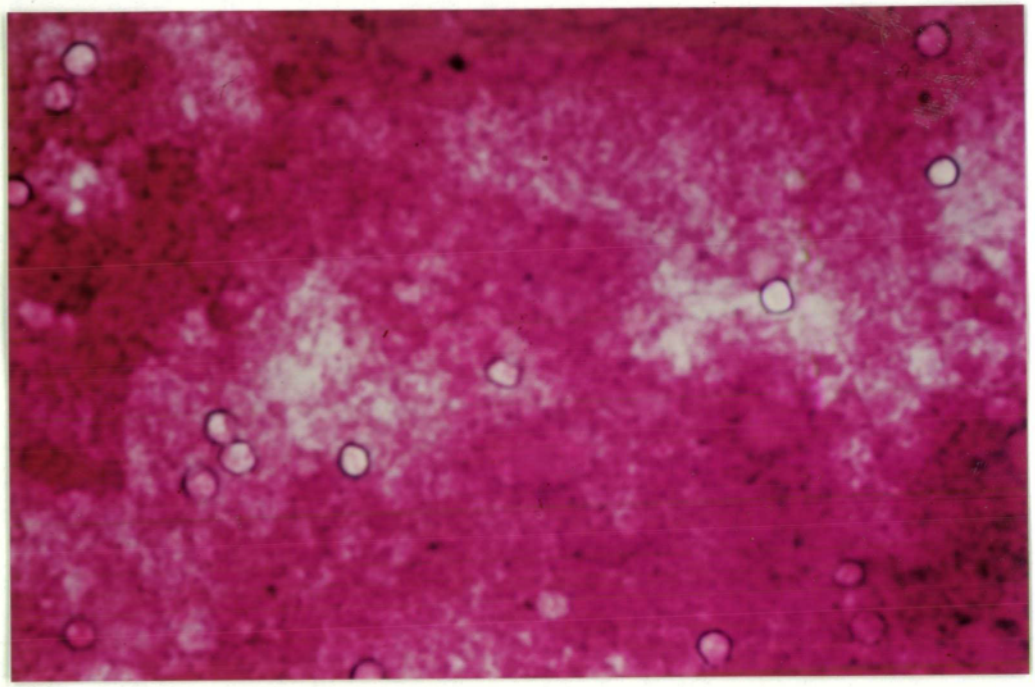


Figure 5. *Cryptosporidium* oocysts. Carbol fuchsin negative stain. x400

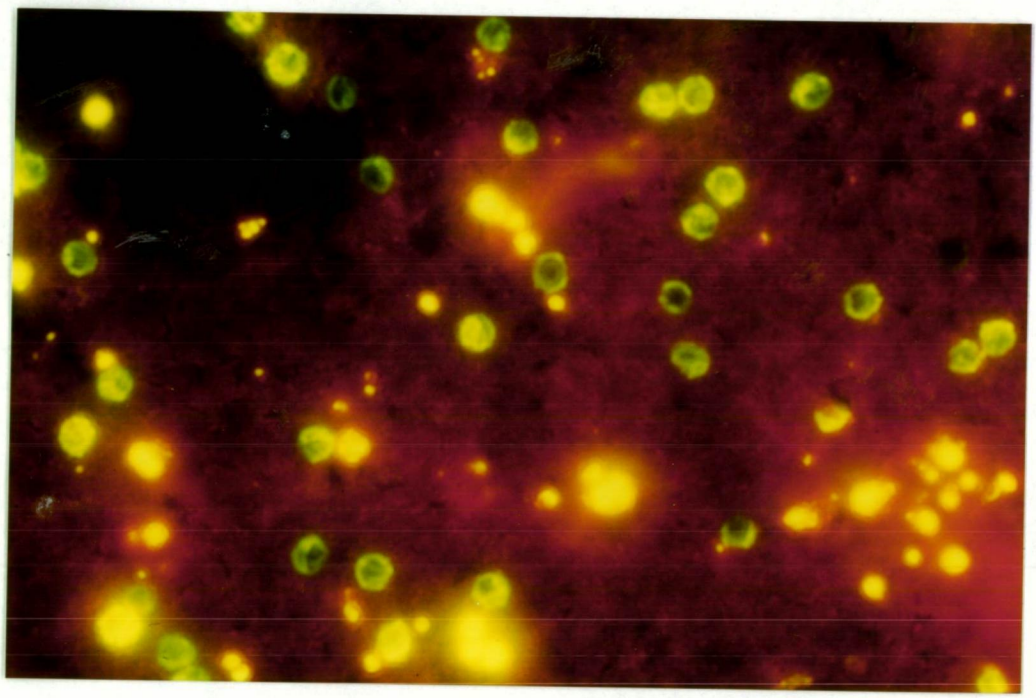


Figure 6. *Cryptosporidium* oocysts. Auramine / carbol fuchsin stain. x400.

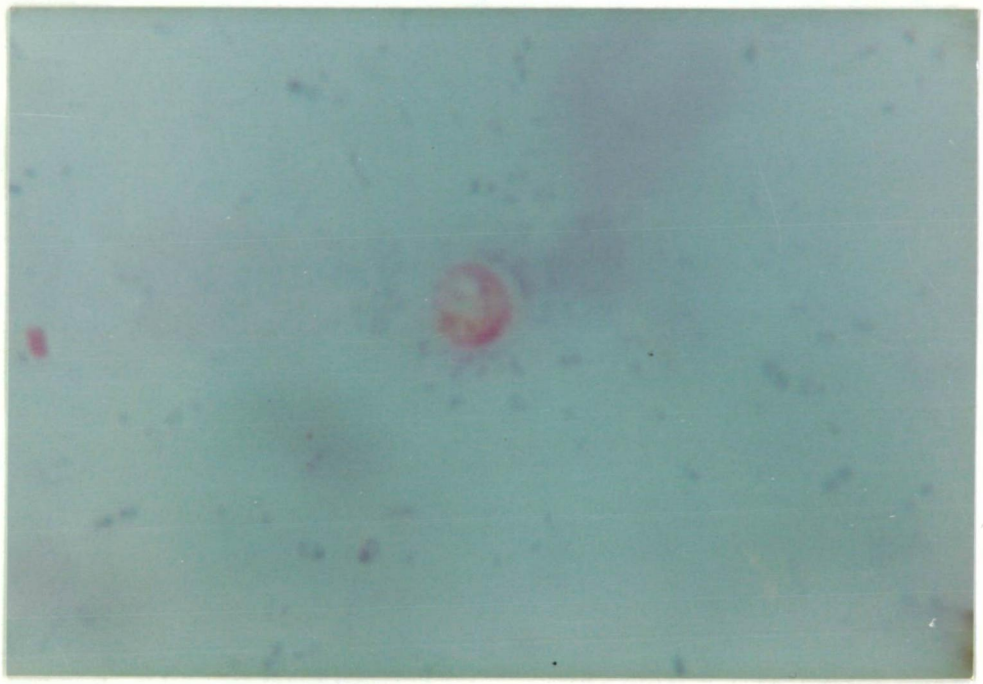


Figure 7. *Cryptosporidium* oocysts. Safranin-methylene blue stain. x1000.

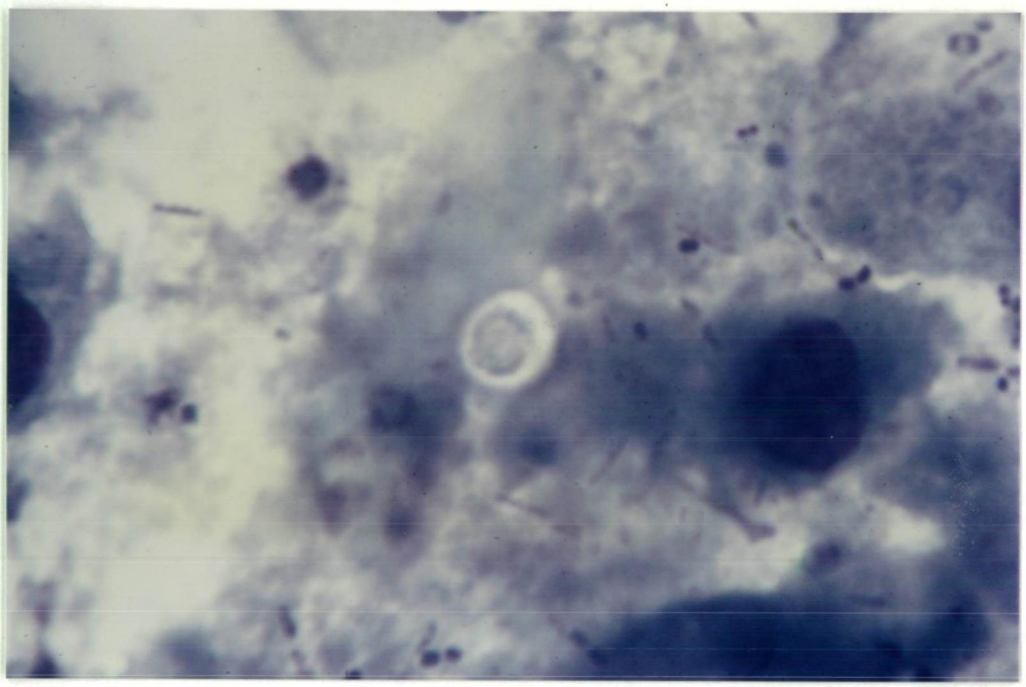


Figure 8. *Cryptosporidium* oocysts. Giemsa stain. x1000.

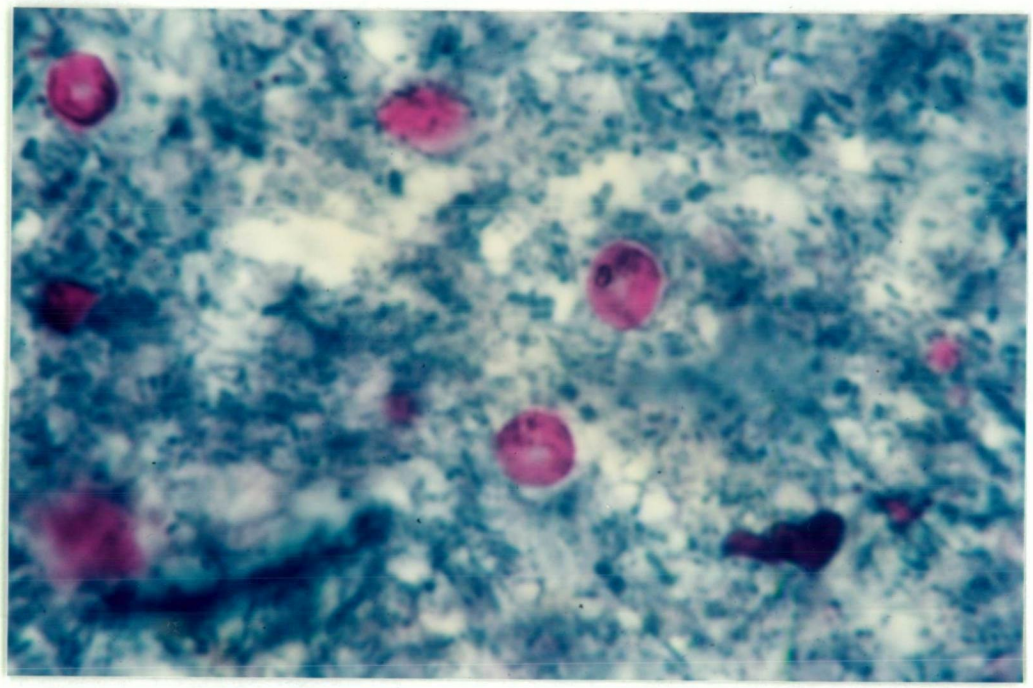


Figure 9. *Cryptosporidium* oocysts. Modified acid-fast stain. x1000.

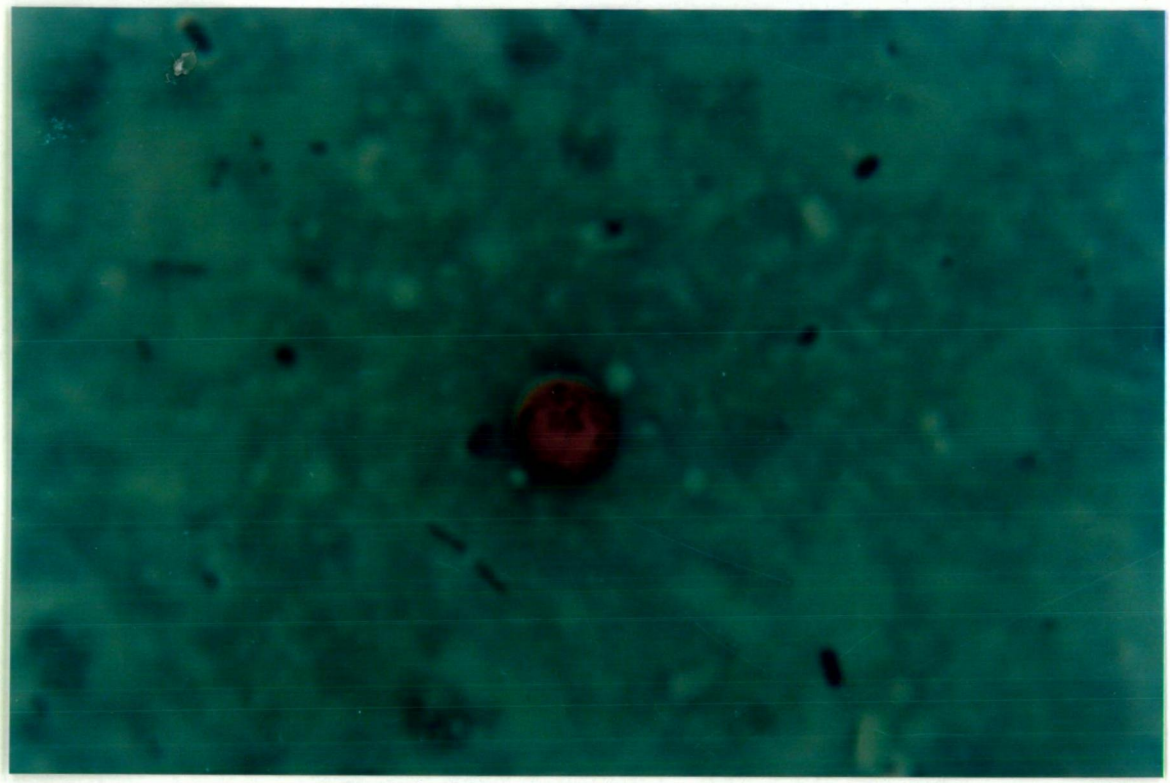


Figure 10. *Cryptosporidium* oocysts. Kinyoun acid-fast x1000

3.3 DISCUSSION

No one staining method for cryptosporidia is completely effective, a view shared by many authors including Ma (1983) and Casemore et al (1985). But, as can be seen from Table 7, the Auramine carbol-fuchsin fluorescent stain appeared the most sensitive, a finding consistent with those of Casemore, Armstrong and Sands (1985). In relation to speed and ease of technique, the carbol-fuchsin negative stain was superior to the others and should be used whenever large numbers of suspicious looking bodies are seen in the faecal wet preparations. It would probably not be adequate where the numbers of parasites present were small.

The safranin-methylene blue stain was disappointing even though workers such as Baxby et al, (1984a) and Bogaerts et al (1984) found it to be more sensitive in their studies. The oocysts appeared pale orange and were relatively difficult to see in most cases. Also, the number of oocysts staining appeared to be fewer than when stained by the carbol-fuchsin or fluorescent techniques.

The Giemsa stain proved to be very insensitive and, as can be seen from the photomicrographs, most of the oocysts did not stain at all, appearing merely as empty objects. Probably for this reason this method is no longer widely

used. Although there is no one completely effective staining technique for routine investigation of faecal samples for *Cryptosporidium*, the findings of this assessment correlate well with the findings of Casemore, Armstrong and Sands (1985) in that the Auramine carbol fuchsin stain, with the option of an acid-fast technique to confirm, is the best system available for routine screening.

4 CRYPTOSPORIDIOSIS IN TASMANIA

The reported incidence of cryptosporidiosis in Tasmania has increased dramatically since the first documented case in 1984 by McColl and Mooney. Launceston and surrounding areas have reported more than 90 cases since then while Hobart only reported their first case in January 1988 and the North-west region has had no reports todate. This study will report the surveillance of the incidence of cryptosporidiosis in the north of Tasmania. Patient studies of some of the more interesting cases will be also be reported along with an attempt to discover the epidemiology of the organism in Tasmania. Seasonal incidence and age related differences will be investigated as well as zoonotic associations.

During the 30 months from July 1986 to December 1988 faecal samples from 3405 patients were examined for the presence of *Cryptosporidium* oocysts using direct and concentration techniques and special stains. The faecal samples examined were routine specimens submitted to the laboratory for testing for gastrointestinal pathogens. All patients with cryptosporidiosis were further assessed as to age, residential location, time of year, history of exposure as well as ingestion of raw milk etc, and any other potential predisposing factors such as immunocompetence.

4.1 MATERIALS AND METHODS

From the initial evaluation studies of available techniques for identification of *Cryptosporidium* the materials and methods used are as follows:-

The samples were first examined for *Cryptosporidium* and other parasites in a direct wet preparation using sucrose flotation (Figure 11) and iodine. They were then processed using the standard Formalin-ethyl acetate concentration technique utilising the FPC parasite concentrator (Figure 12), (Evergreen Scientific, Los Angeles, Calif.), according to the manufacturers instructions. Casemore's modification of the auramine-phenol fluorescent method (Figure 6) was used for staining all concentrates as it was found in the earlier study to be a simple, rapid, and sensitive method for the detection of *Cryptosporidium* oocysts. The Kinyoun acid-fast method (Figure 10) described by Ma & Soave, 1983 was rarely used to stain any doubtful samples.

In addition, stool samples were routinely cultured for *Salmonella*, *Shigella*, *Aeromonas* and *Campylobacter*. Samples from children aged less than 2 years were routinely tested for rotavirus, initally using the Slidex Rota-kit latex test (Biomerieux;CSL) but later changing to an Enzyme-Linked Immunosorbant Assay (Kallestad).

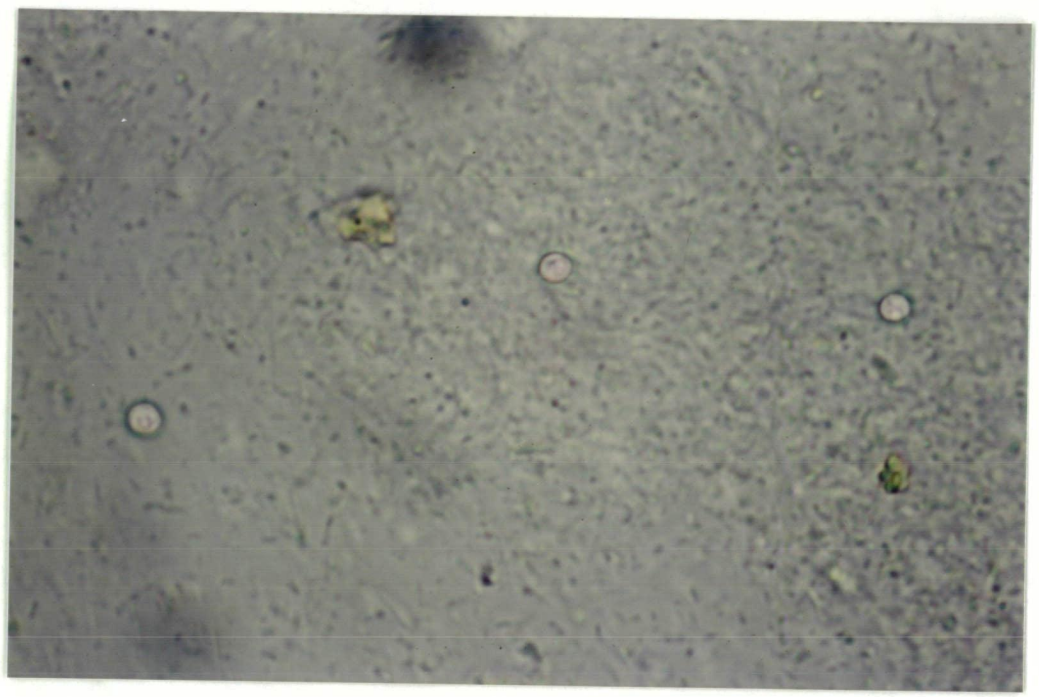


Figure 11. *Cryptosporidium* oocysts. Sucrose floatation x400

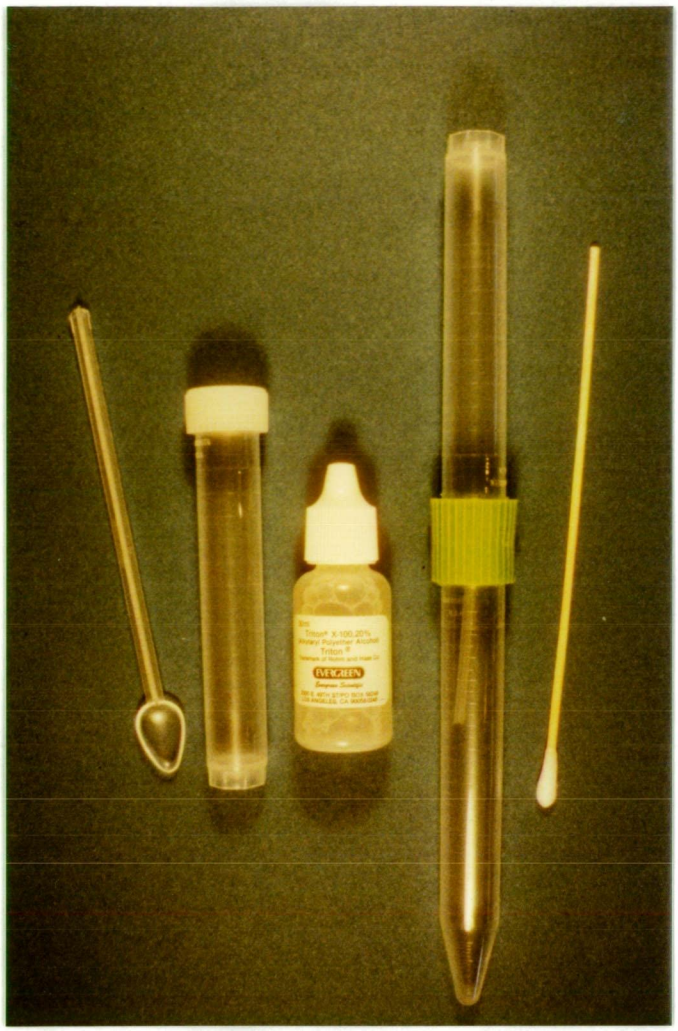


Figure 12. Faecal Parasite Concentration equipment (Evergreen Scientific).

4.2 RESULTS

The results of the survey, including all the other significant organisms isolated, is summarised in Table 8.

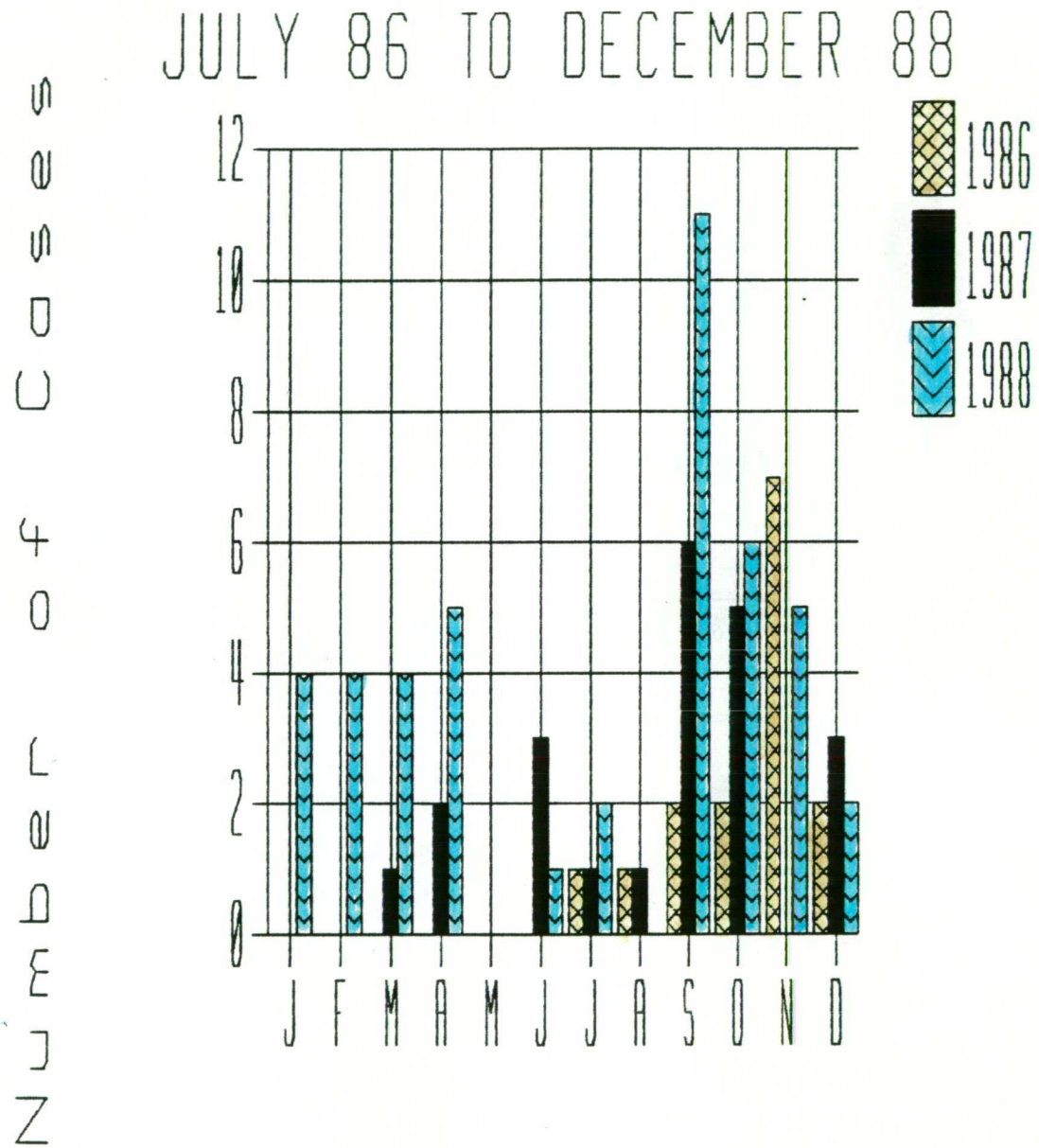
	No. of cases	% of total
Campylobacter jejuni	367	10.7
Cryptosporidium sp.	81	2.0
Giardia lamblia	74	1.8
Rotavirus *	58	1.4
Salmonella sp.	57	1.4
Aeromonas hydrophila	19	0.5
Blastocystis hominis	12	0.3
Clostridium difficile $	6	0.15
Entamoeba coli	6	0.15
Endolimax nana	4	0.1
Hookworm	4	0.1
Entamoeba histolytica	2	0.05
Strongyloides sp.	1	0.02
Plesiomonas shigelloides	1	0.02
Trichuris trichiura	1	0.02
Ascaris lumbricoides	1	0.02
Shigella sonnei	1	0.02
TOTAL	695	

Table 8. Summary of the organisms identified in the survey of 3405 patients.
* only tested for on patients aged ‹2yrs.
$ only tested for on request.

Over the 30 month period 81 cases (2.0%) of cryptosporidiosis were identified. The monthly incidence is shown in Figure 13. The total incidence was second only to that of *Camplylobacter jejuni* and was slightly higher than *Giardia lamblia*. Figures 14, 15, & 16 show the comparative prevalence year by year of the three major organisms identified during the survey period.

The age distribution of cases shown in Figure 17 shows a clear preponderance of children under 5 years of age with cryptosporidiosis. The next most commonly infected group is in the 6 to 15 year age range followed by the 26 to 35 year range. Patients aged 36 years or more showed a significantly lower incidence of infection in this study.

Case studies are summarised in Table 9. Expanded case histories of the more interesting patients, including those from whom blood samples were obtained for immunological studies, are recorded in Appendix 7.4.



MONTHLY TOTALS

Figure 13

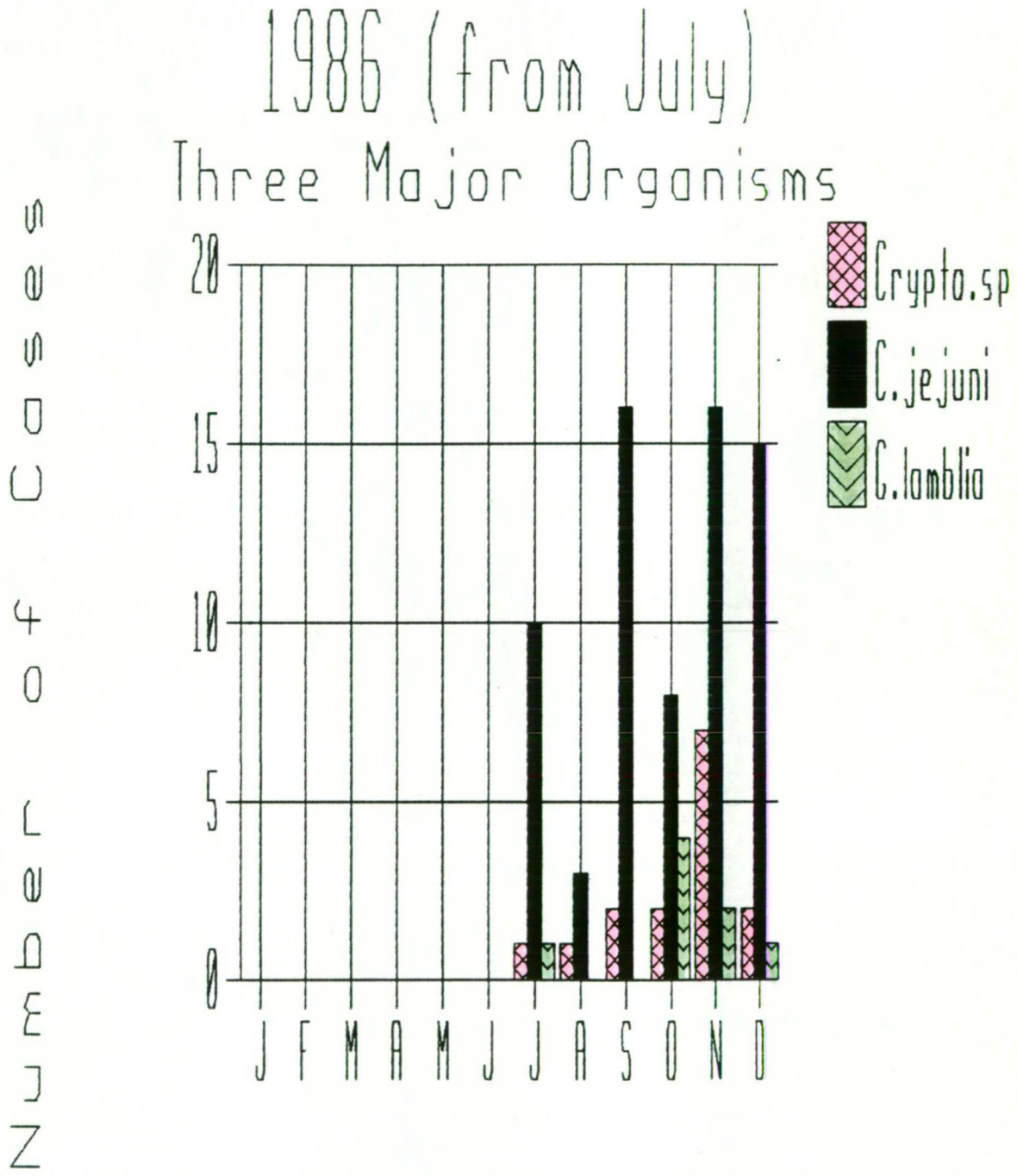
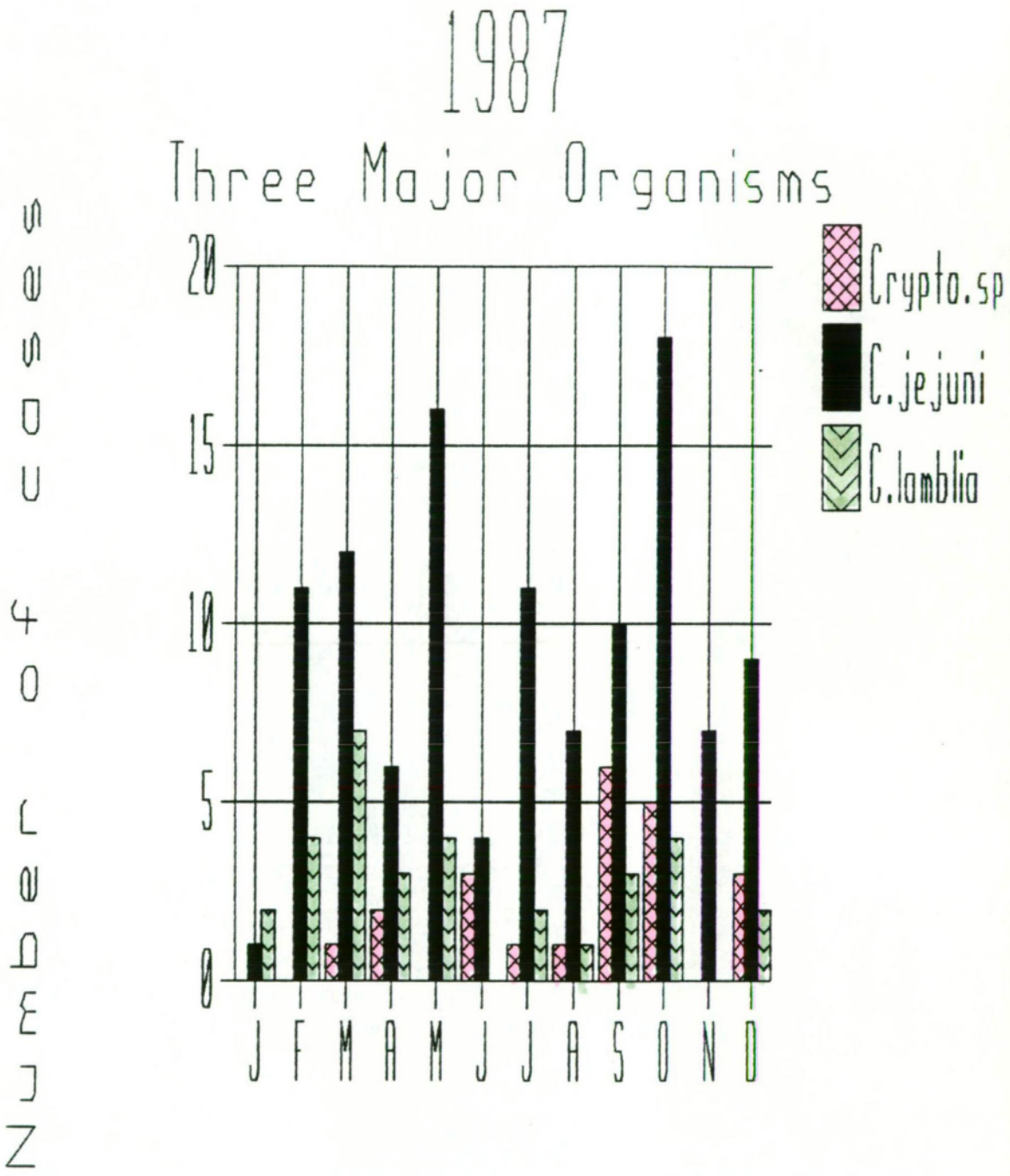
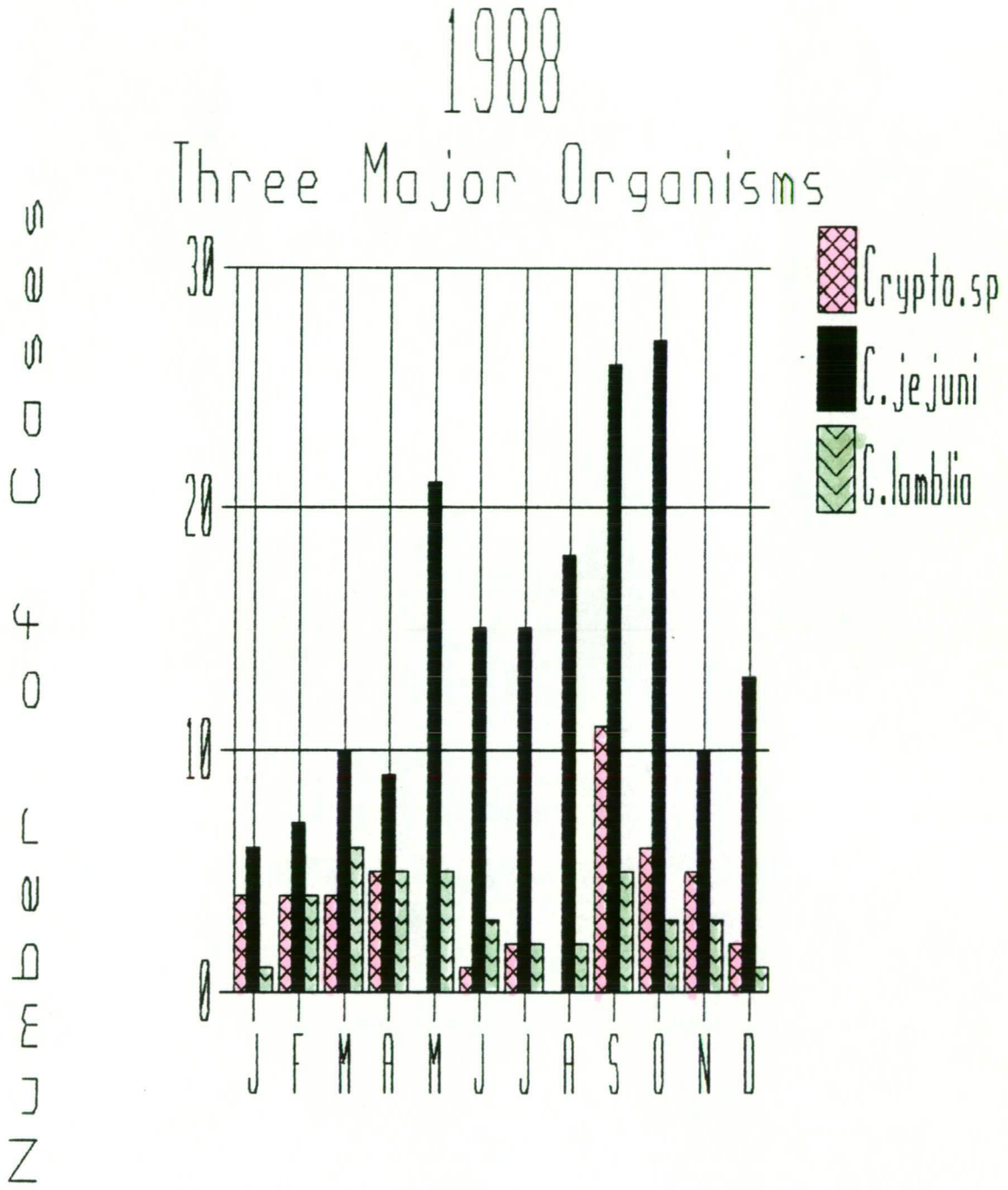

1986 (from July)
Three Major Organisms
Number of Cases
20
15
10
5
0
J F M A M J J A S O N D
Crypto.sp
C.jejuni
G.lamblia
MONTHLY TOTALS

Figure 14



Monthly Totals

Figure 15



Monthly Totals

Figure 16

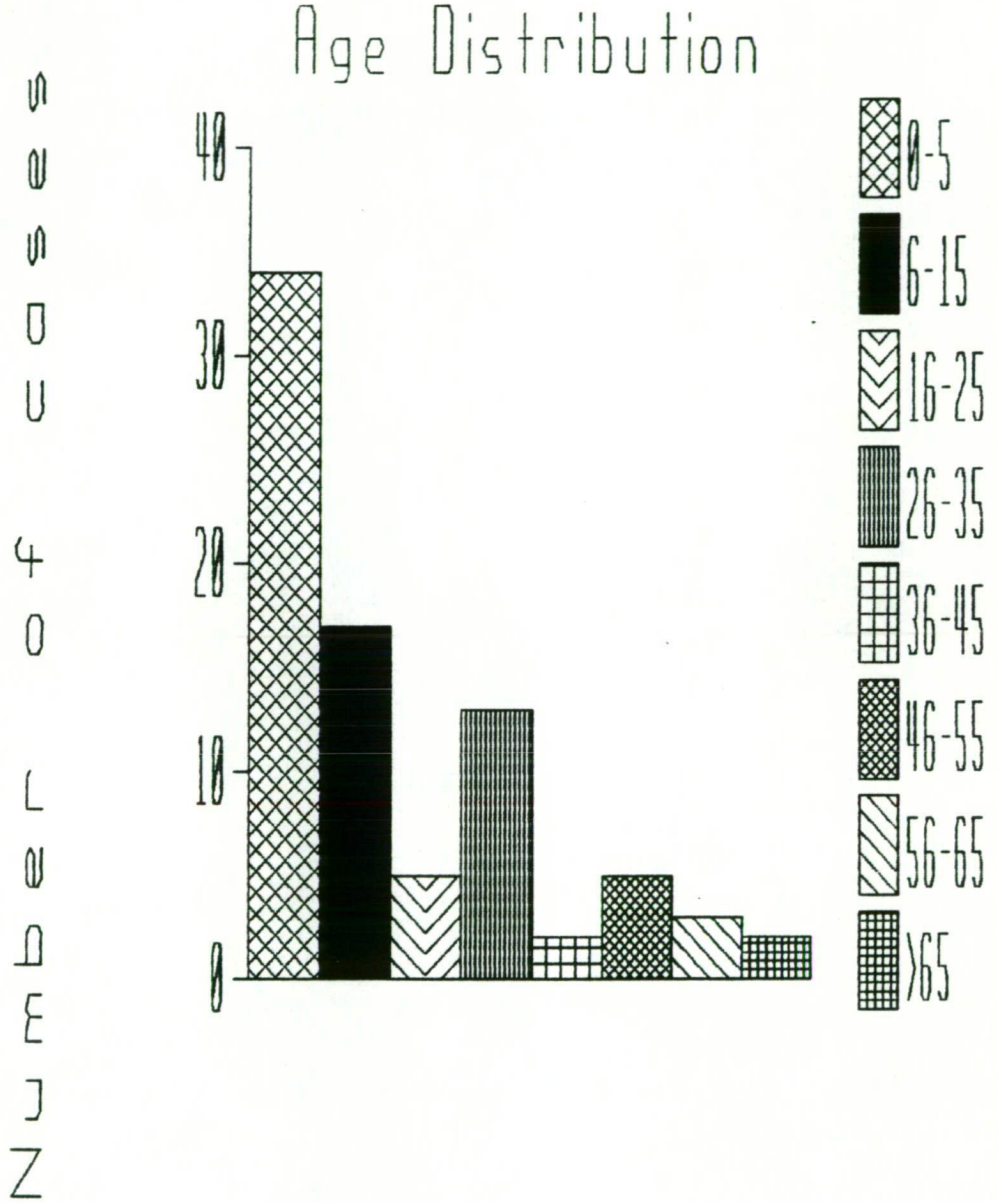

Age Distribution
Number of cases
40
30
20
10
0
0-5
6-15
16-25
26-35
36-45
46-55
56-65
>65
Age in Years

Figure 17

CASE NO.	SEX	AGE	MONTH	AREA	SYMPTOMS	COMMENT
1	F	27	July	Legana	D V A L	Raw milk
2	M	2	Aug	Westbury	D	
3	F	4	Sept	Westbury	D P L	
4	M	2	Sept	St Helens	D	
5	M	9	Oct	Legana	P	
6	M	4	Oct	Launceston	V	
7	M	31	Nov	Launceston	H D P F	Milking cows
8	F	6	Nov	Launceston	D P	Mother +ve too
9	F	35	Nov	Launceston	D	person-person
10	M	6	Nov	Launceston	H D	raw milk
11	M	4	Nov	Launceston	D	
12	F	2	Nov	Deloraine	D	raw milk
13	M	48	Nov	Launceston	D V A L	raw milk
14	F	27	Dec	Legana	D V A P F	
15	M	29	Dec	Launceston	D	
16	M	9	March	Dunorlan	D	dairy farm
17	M	2	April	Derby	D	related to 18
18	M	2	April	Derby	D	related to 17
19	M	29	June	Longford	D P A L	raw milk
20	M	32	June	Launceston	D	
21	F	49	June	Launceston	H D V A P	raw milk
22	F	1	July	Scottsdale	D P F	raw milk
23	F	2	Aug	Devon Hills	D	
24	F	23	Sept	Cleveland	D	
25	M	5	Sept	GeorgeTown	D V A L F	raw milk
26	M	10	Sept	Launceston	D	raw milk
27	F	2	Sept	Deloraine	D V A F L	calves
28	F	2	Sept	Scottsdale	D P	raw milk
29	F	8	Sept	Scottsdale	D P	
30	M	1	Oct	Deloraine	D	brother of 12
31	F	49	Oct	Launceston	D	been overseas
32	M	55	Oct	Launceston	D	
33	F	4	Oct	Deloraine	D P V L A	raw milk
34	F	3	Oct	Deloraine	D	
35	M	26	Dec	Launceston	D	meat works
36	F	2	Dec	Launceston	D	
37	M	1	Dec	Launceston	D	
38	F	7	Jan	Launceston	D V L A F	
39	M	1	Jan	Launceston	D	C.jejuni
40	F	1	Jan	Hillwood	D	
41	M	13	Jan	Launceston	D	
42	M	7	Feb	Dilston	D	C.jejuni
43	F	2	Feb	Launceston	D P L A F	
44	F	17	Feb	Windemere	V D P F	asthma camp
45	M	12	Feb	Launceston	P D A L	camping
46	F	1	Mar	Deloraine	D	
47	M	27	Mar	Launceston	D V L A F	
48	F	4	Mar	Scottsdale	D	G.lamblia
49	M	2	Mar	Launceston	D	

cont.....

cont.....

50	F	3	April	Launceston	D				
51	M	10	April	Port Sorrel	D	V			Died from AIDS
52	F	65	April	Launceston	D	N			?sheep manure
53	M	8	April	Launceston	D				
54	F	41	April	Launceston	H	V	D		
55	F	2	June	Launceston	D				
56	M	10	July	Devon Hills	D	P	N		
57	M	23	July	Hadspen	D				Raw Milk
58	M	2	Sept	Scottsdale	V				C.jejuni
59	F	30	Sept	Westbury	H	P	D		
60	F	15	Sept	Westbury	D				C.jejuni,milk
61	M	3	Sept	Launceston	D	V			
62	M	63	Sept	Launceston	D				
63	M	65	Sept	Scottsdale	D				Raw milk
64	M	38	Sept	Deloraine	D				
65	F	29	Sept	Launceston	D				
66	M	35	Sept	Launceston	D				Raw milk
67	M	34	Sept	Georgetown	D	A	L		
68	F	52	Sept	Longford	D	F			
69	F	1	Oct	Deloraine	D				Raw milk
70	M	3	Oct	Deloraine	D				
71	F	27	Oct	Dilston	D	F			Raw milk
72	M	1	Oct	Georgetown	D				Rotavirus
73	M	8	Oct	Deloraine	D				Raw milk
74	M	67	Oct	Launceston	D	P	A	L	Campylobacter
75	M	6	Nov	Launceston	D				
76	M	2	Nov	Deloraine	D				Raw milk
77	M	3	Nov	Scottsdale	D				Raw milk
78	F	74	Nov	Launceston	D				
79	M	5	Nov	Launceston	D				farm visit
80	M	20	Dec	Hadspen	D				
81	F	9	Dec	Beaconfield	D	L	F	A	raw milk

Table 9. Summary of the case studies of the survey. D=Diarrhoea, V=Vomiting, H=Hospitalised, L=Lethargy, F=Fever.

4.3 DISCUSSION

The survey showed a 2.0% incidence of cryptosporidiosis, which compares quite well with other studies in developed countries including mainland Australia, although it is difficult to relate directly to any one study due to differences in the methods of assessment. Some workers selected out samples that had abundant small organisms or bodies for their surveillance (Jokipii et al, 1983), hence the high percentage of positives for a developed country (9.1%). Studies of unselected samples, such as that of Palmer (1986), who found an incidence of 1.2%, at Moree N.S.W., showed results relating much more closely to those recorded in the present study.

Another factor that affects correlation of results are the type of patient surveyed. Studies done in hospitals and especially childrens hospitals are more likely to record a higher prevalence. For instance the study by Tzipori et al (1983) in the Royal Melbourne Childrens Hospital showed that 4.1% of the patients were excreting oocysts. It is interesting to note that out of all the patients with cryptosporidiosis in this Tasmanian survey, 60% were children or young adults.

Another factor making correlation difficult was

demonstrated by Lumb et al (1985) who surveyed Adelaide's population for 4 years and detected an average 0.12% infection rate of *Cryptosporidium* in 9056 stools examined. They then sampled 94 Aboriginal children from Alice Springs and found a positive rate of 9.6% which is extremely high for a country classified as developed. This once again emphasizes the particular problems with Aboriginal health in Australia that has been documented recently (Goldsmid. 1988).

During the survey period a high number of cases of *Salmonella* were encountered and this was mainly due to 3 presumed outbreaks of food poisoning. The increased number of cases of rotavirus seen during the study is probably due to the adoption of a more sensitive method for detection of the virus antigen, i.e ELISA instead of latex agglutination.

Seven patients (5.7%) with cryptosporidiosis also had concomitant infections with other organisms including *Campylobacter jejuni* (5), *Giardia lamblia* (1) and rotavirus (1). In most cases, both organisms in these patients were probably obtained from the same source.

The nature of mixed infections may suggest possible common epidemiology. Many surveys showed other recognised enteric pathogens such as *Campylobacter jejuni*, *Giardia*

lamblia, *Salmonella* sp. and rotavirus to name a few (Jokipii et al, 1983; Bogaerts et al, 1984; Ratnam, 1984; Wolfson et al, 1985; Casemore, 1986; Palmer, 1986; Carter, 1986; Biggs et al, 1987).

One interesting aspect of this study is that many of the cases of cryptosporidiosis detected to date had a rural connection of some sort, a factor that is confirmed by other workers in Launceston. Animal contact, especially young animals seems to be an important factor involved in many of the cases.

Unpasteurised milk is a possible source of infection as a surprising number of patients had recently consumed raw milk and often made a habit of doing so. These findings are consistent with those of Palmer, who in 1986 found 10% of cryptosporidiosis patients had recently consumed raw milk. More so, continued consumption of raw milk may lead to reinfection in some cases.

Cases eight and nine probably represent the first recorded instances of definite person to person spread of *Cryptosporidium* in Tasmania. This was highlighted by the infection of a child from a probable animal source and the resultant transmission to the child's mother. Also, although not proven by identification of *Cryptosporidium* oocysts in her stools, the mother of case sixteen was found

to have antibodies in her blood consistent with recent exposure to *Cryptosporidium*.

There also appears to be a seasonal trend, as seen in Figure 13, and this has been previously described in other parts of the world during spring and early summer (Wyllie, 1984; Hunt et al, 1984; Current, 1985; Montessori and Bischoff, 1985; Baxby and Hart,1986; Carter, 1986; Palmer, 1986). The highest incidence in this study occurred during the months of September to November (late spring to early summer) with another less noticeable peak around March to April (late autumn).

The higher rate of cryptosporidiosis in children in surveys of unselected patients has been documented. Palmer & Biffin (1986) found a 56% incidence overall in children aged 15 years or less. Also, study by Casemore (1987a) showed a 69% incidence in children of this age group (Figure 4, page 19). This survey found a 60% incidence of cryptosporidiosis in children aged from 0 to 15 years and reasons for this are unclear. The self-limiting nature of the disease in immunocompetent patients may well make it more likely for children to be presented for diagnosis of their symptoms earlier than adults. Many general practitioners interviewed said their usual advise to most adult patients with the common symptoms of cryptosporidiosis was "If you're not better in 4 days, come

back and see me. Sounds like you've got a touch of 'gastric flu' to me".

Recently, sporadic cases of cryptosporidiosis have been reported in the Hobart area although not of the same magnitude as in Launceston. The fact that the organism is not routinely sought in some laboratories must explain the low incidence in other areas of Tasmania. Laboratories only performing saline and iodine wet preps. are most apt to miss identification of *Cryptosporidium*, as it is best seen in a Sheather's sucrose flotation preparation. Also the rural environs and climate of Launceston may lend themselves well to survival and transmission of the organism. As can be seen in Figure 13, the increased incidence of *Cryptosporidium* in early summer could be related to the preceding month's usual warm, wet weather and abundant lush green grass. Further, the fact that spring heralds the arrival of many young animals could contribute to the higher incidence in early summer.

Overall, prevalence studies done here have indicated that *Cryptosporidium* must be regarded as a unique cause of diarrhoea in this state. The scarcity of recordings from the South and other areas probably represents a lack or awareness or of technique selection for routine studies.

There would seem to be a need to incorporate routine

examination for *Cryptosporidium* in diagnostic medical laboratories and especially if effective chemotherapy becomes available. Even without the availability of effective drug treatment, however, the ability to identify *Cryptosporidium* as the cause of the diarrhoea can be important in the control of spread, especially in the light of increasing evidence to incriminate animal sources of primary infection and the encroaching epidemic of AIDS. Continued surveillance is necessary before an overall picture can be established.

5 HUMAN IMMUNE RESPONSE

Until now, no research into the human immune response to cryptosporidiosis has been carried out in Tasmania. The demonstration of an immune response in the relatively newly recognised infection of human beings is essential for assessing pathogenicity, for diagnostic purposes, and for epidemiological studies. The fact that little or no cryptosporidiosis occurred, or was detected, in other parts of Tasmania warranted further investigation. Thus, once a method was found that showed an immune response to cryptosporidiosis, then random blood samples from other areas in Tasmania could be assessed for the presence of antibodies also.

This study will show that there is a distinct and typical humoral response to infection with *Cryptosporidium* and that most patients tested for antibody response to cryptosporidiosis demonstrated a similar antibody class production.

The random control group of persons not known to have been exposed to *Cryptosporidium* will confirm that cryptosporidiosis does occur in other areas of Tasmania and that the disease is probably considerably more common than has been shown in the past.

5.1 MATERIALS AND METHODS

ANTIGEN MATERIAL

A supply of *Cryptosporidium* oocysts, free of faecal contaminants, is necessary for studies such as in vitro cultivation, production of hyperimmune sera for immunodiagnostic tests, and as antigen in serological immunoassays. Various methods have been described in the literature, including the use of Percoll (Waldman et al, 1986; Casemore, 1987b), Sucrose (Heyman et al, 1986) or a combination of both methods (Arrowood & Sterling, 1987).

Stool samples from all patients infected with *Cryptosporidium* were preserved by mixing with equal volumes of 2.5% potassium dichromate ($K_2Cr_2O_7$) and then stored at 4°C for future use. These stools were pooled and experimentally processed, using both Percoll and sucrose, to determine the best method for purification of *Cryptosporidium* oocysts.

ANTIGEN PURIFICATION TECHNIQUES

Two methods for purification of oocysts, Percoll and sucrose were compared for yield of oocysts and ease of performance. Details of the methods are shown in Appendix 7.3. The Percoll density gradient using concentrations of

1.04g/ml and 1.08g/ml gave the highest yield of oocysts and also the most pure. The density gradient of Percoll using concentrations of 1.017g/ml and 1.03g/ml produced a similar yield to the 1.04/1.08g/ml gradient but contained large amounts of bacteria and faecal debris. The banding patterns of oocysts in the Percoll discontinuous gradient are shown in Figure 18.

The method using the sucrose density gradient proved the least successful due to low oocyst yield and marked contamination of the pellet with bacteria, yeasts, and faecal debris. The ease of performance of the two methods was clear cut. In respect of preparation of reagents, preparation of gradients, and definition of the zone of concentration, the method employing Percoll was far superior in every respect.

The sucrose solutions needed to be maintained at a set low temperature to ensure their inability to mix with the other concentrations in the gradient. This meant that the gradient had to be spun down in a refrigerated centrifuge. It was also found to be extremely difficult to layer the two sucrose solutions as there was always some degree of mixing no matter how delicately the operation was performed. The different densities of Percoll appeared much more resistant to mixing than the sucrose solutions and therefore formed a better defined gradient.

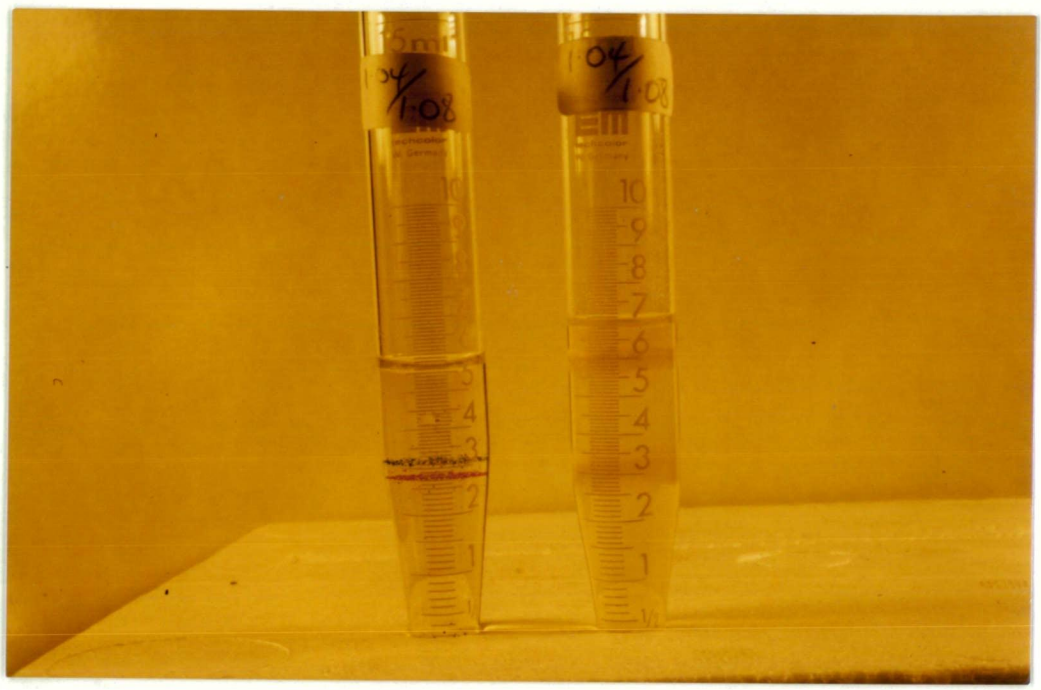


Figure 18. Percoll density gradients showing density beads on left and faeces in right.

Although the size of the oocyst (4-8um) makes complete purification of oocysts from faeces difficult, the Percoll method provides preparations of sufficient purity for immunological studies, such as the immunofluorescent-antibody labelling of *Cryptosporidium* oocysts, without excessive interference from extraneous antigenic material.

ANTIBODY MATERIAL

Samples of serum from 11 patients diagnosed as having cryptosporidiosis were stored frozen for later testing. Samples from as early as 5 days to as late as 554 days post infection were obtained from different patients. Four patients had more than one sample taken. In two cases, pre-infection blood samples were also available. Most of the patients studied for antibody response were adults, due to the difficulty in obtaining blood samples from children.

Control samples of 10 rural and 10 urban normal, uninfected people from each of the North-west coast, Hobart, and Launceston areas were also collected for testing. The control samples were deliberately collected during the annual peak incidence period between September and November. Two sera from patients with known positive Toxoplasmosis IgG and IgM serology were also tested to eliminate any cross-reactivity by this organism with the

assay.

ANTIBODY DETECTION TECHNIQUE

The method used for detection of *Cryptosporidium* antibodies in serum was a standard indirect immunofluorescent technique modified by experimentation to suit the assay. Fluorscein iso-thio-cyanate (FITC) anti-human globulin conjugates used were sheep anti-human globulin (AHG), sheep anti-IgG, sheep anti-IgA and sheep anti-IgM (Wellcome, Australia). The antibody detection method is summarised in Figure 19 and detailed in the Appendix 8.3.

Doubling dilutions of serum samples from 1 in 20 to 1 in 1280 were prepared in small plastic test tubes using phosphate buffered saline (PBS) pH 7.3 as the diluent. Test samples and antigen coated slides along with the class of FITC/antiglobulin conjugate used were coded on a worksheet and then later decoded for analysis of results.

Slides were examined immediately using an Olympus Flourescence Vertical Illuminator with x40 objective.

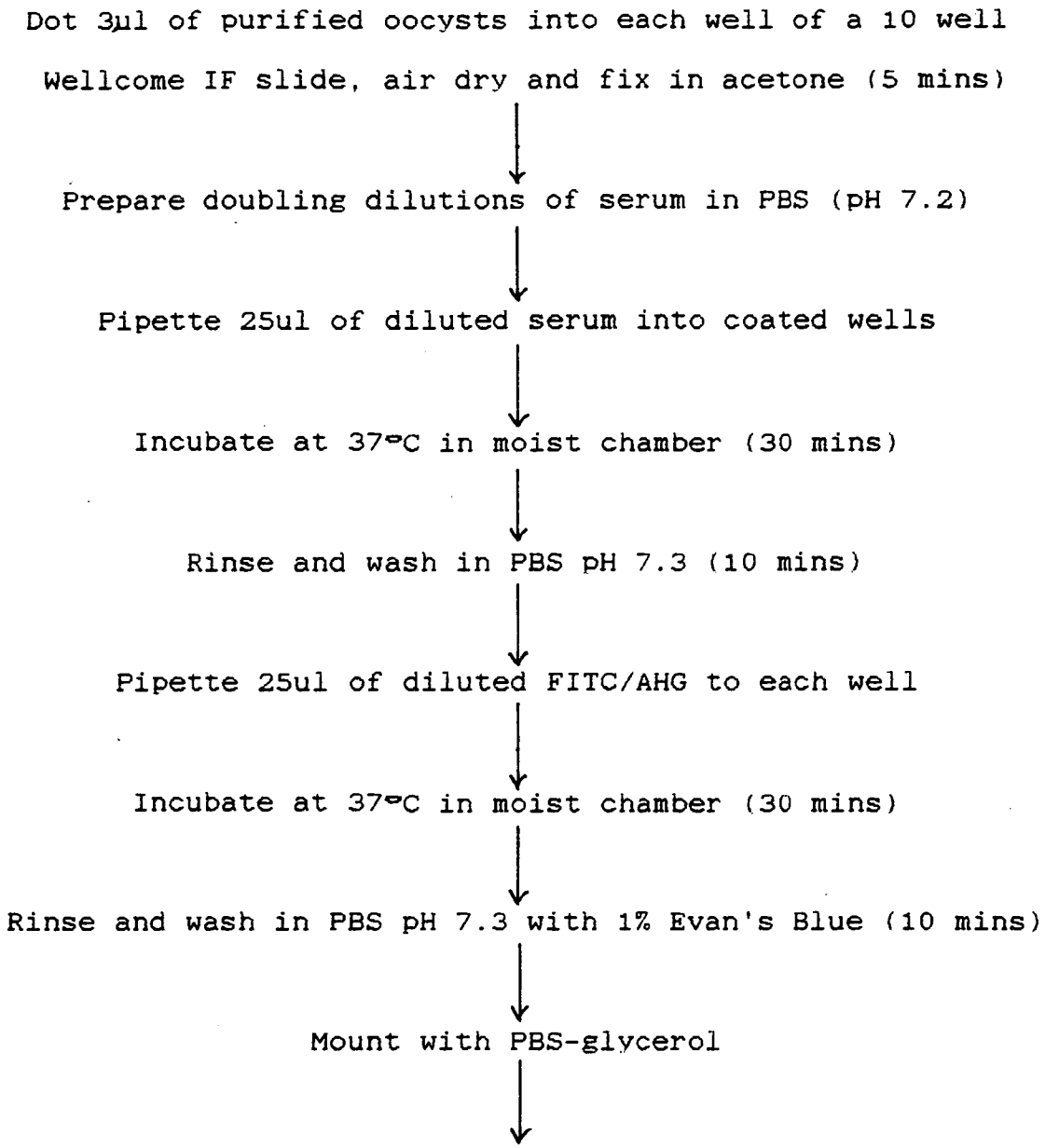


Figure 19. Diagram of the indirect immunofluorescent method.

5.2 RESULTS

The *Cryptosporidium* oocysts fluoresced brightly with positive samples (Figure 20) but only faintly or not at all with negative samples and the conjugate alone. Only the outer case of the oocyst stained; sporozoites within could not be seen. The results of the AHG antibody study from case and control groups are shown in Table 10 and Figure 20A shows graphically the positive levels of AHG in the three groups. The samples clearly showed a bimodal distribution of titres between case and control groups but with little difference between urban and rural groups.

Titre	Samples from 30 random urban controls	Samples from 30 random rural controls	Samples from confirmed cases (varying time after onset)
1280	0	0	2
640	0	0	2
320	0	1	6
160	2	1	3
80	2	2	3
40	3	3	2
20	3	6	1
<20	20	17	0

Table 10. Human antibody response to *Cryptosporidium* tested with AHG/FITC.

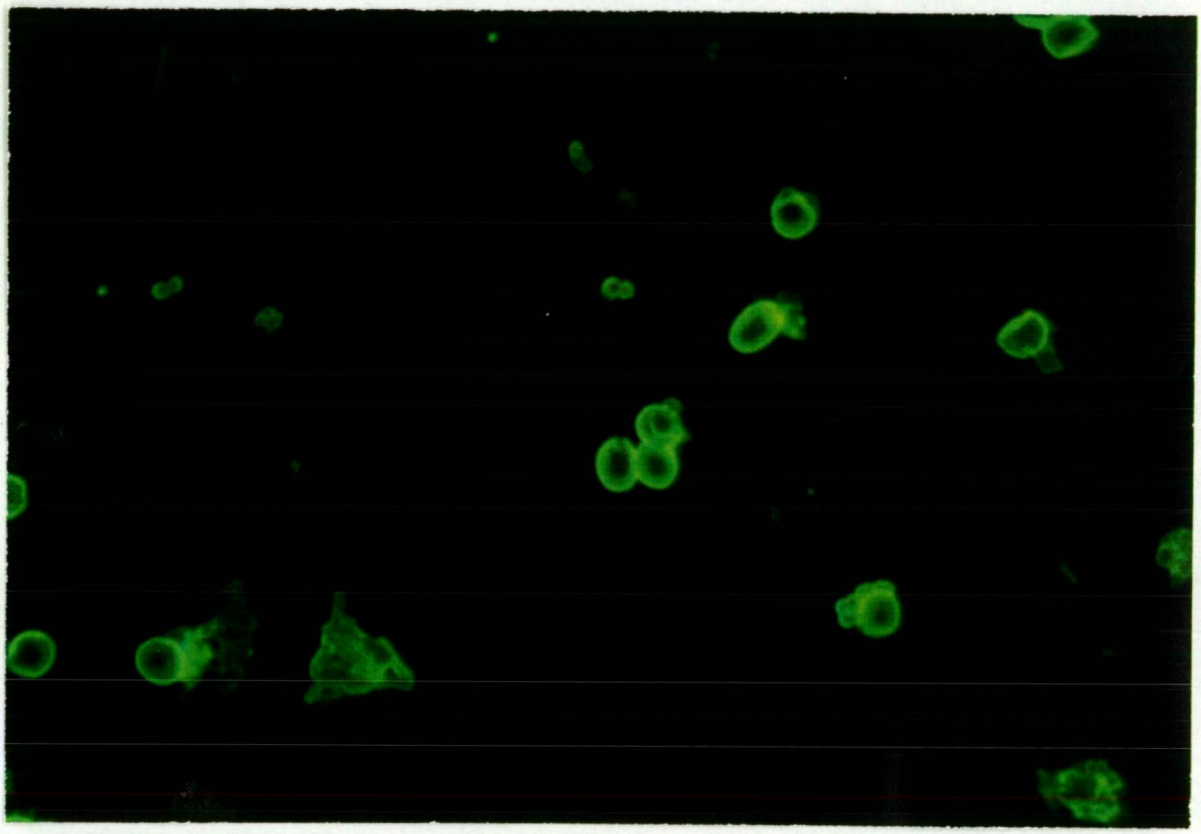


Figure 20. Photomicrograph of *Cryptosporidium* oocysts in positive IIFA test samples. x400

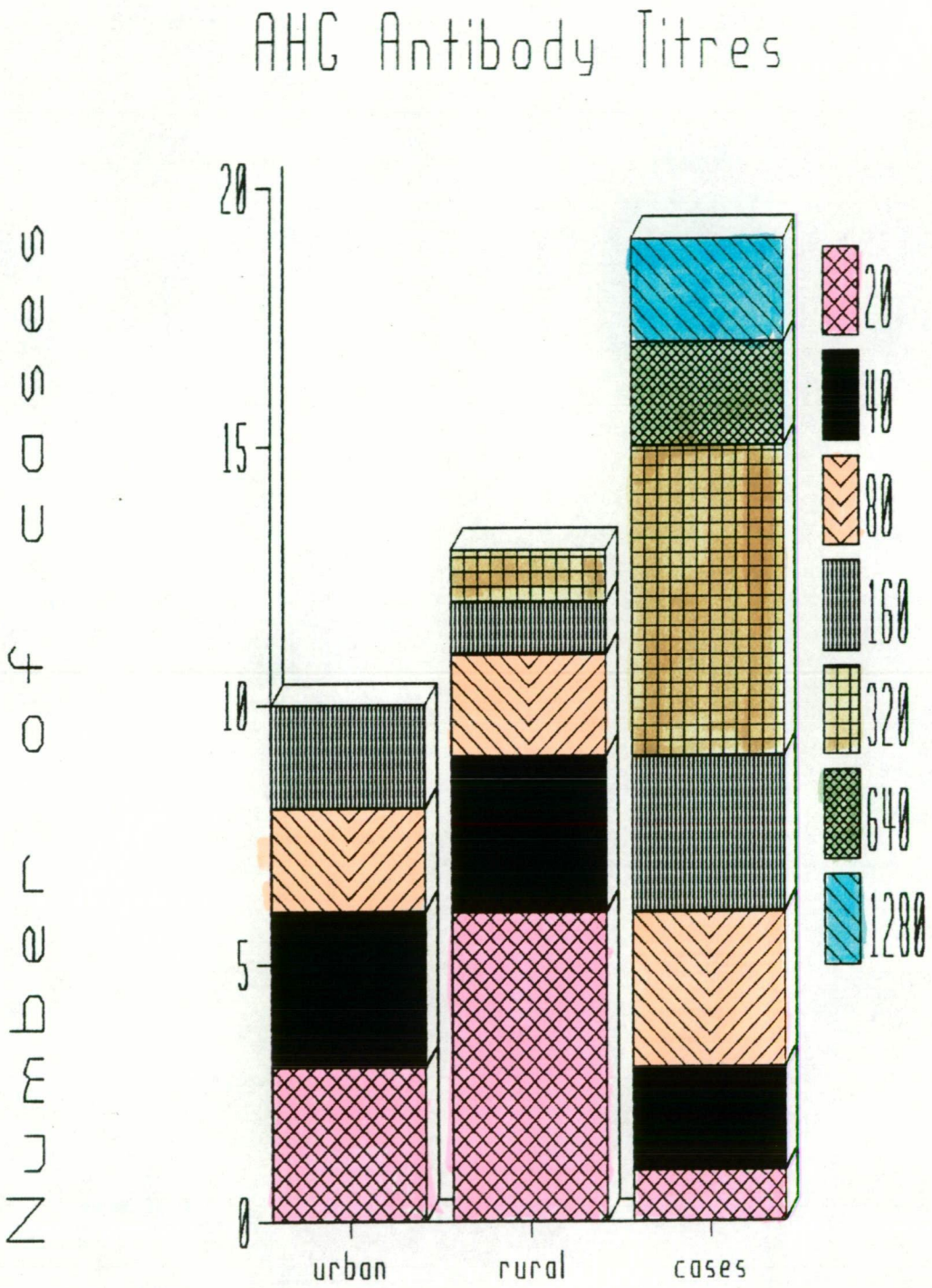

AHG Antibody Titres
Number of cases
20
15
10
5
0
urban
rural
cases
20
40
80
160
320
640
1280

Figure 20A

The samples initially positive (› or = to 20) by AHG were further tested to determine the various concentrations of class-specific immunoglobulin. Of the controls, one urban patient from the North-west coast had raised IgG, IgM and IgA while all the other controls (rural and urban) showed raised IgG only.

The number of random control group samples with antibodies to *Cryptosporidium* from the different areas of Tasmania is shown in Table 11.

Number of samples with antibodies to *Cryptosporidium*

	Launceston	Hobart	North-west	Total
Urban	5	3	2	10
Rural	5	4	4	13
Total	10	7	6	23

Table 11. Patients with AHG antibodies to *Cryptosporidium* from different areas of Tasmania.

All serum samples available from known cases were also tested for the three different classes of antibodies. Details of studies of surveyed patients are documented in Appendix 8.4. The results for the seven patients from whom only a single sample was available are shown in Table 12 and Figure 21. As the samples were taken at varying times post infection, it was possible to follow the rise in titre

of the different classes of antibody. In all but one case the IgA class of antibody was the first to rise and the exceptional case had a massive IgG response which was only one dilution more than the IgA response.

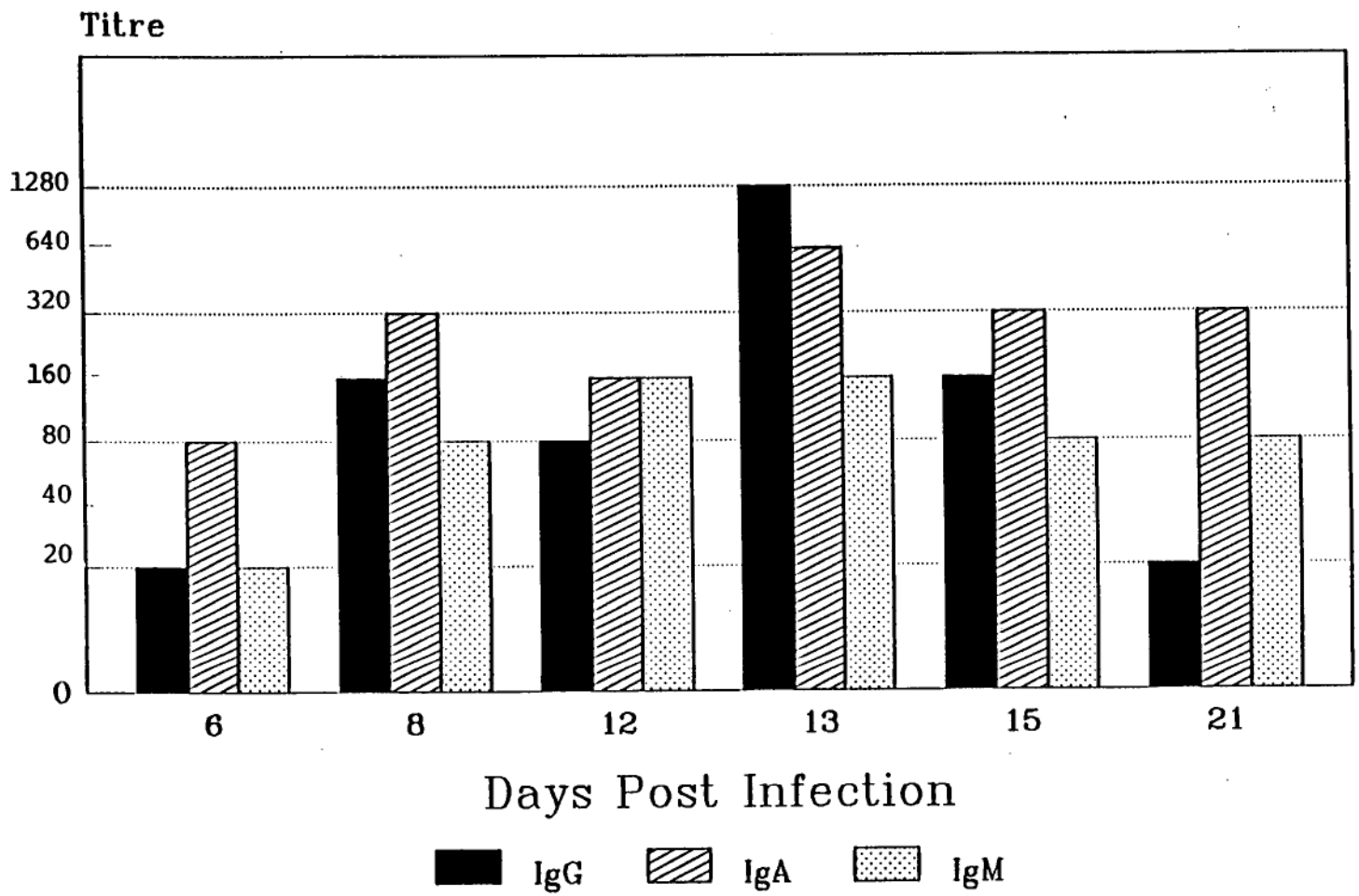
CASE NO.	AGE	AFTER ONSET	TITRE IgG	IgA	IgM
8	31	6	20	80	20
20	29	8	160	320	80
65	29	12	80	160	160
55	41	13	1280	640	160
17	10	15	160	320	80
7	6	21	<40	320	80
52*	10	42	20	20	<20

Table 12. Antibody titres of patients with single samples.
* patient with AIDS

The four multiple samples from confirmed cases, taken at widely varying intervals after infection were, tested for IgG, IgA, and IgM antibodies to *Cryptosporidium*. Results are shown in Table 13 and Figures 22-25. All showed a rise in titre of each class of immunoglobulin tested. In most cases there was an initial rise in IgA, followed by IgG and IgM. This was then followed by falling off of the IgA and IgM while the IgG remained elevated.

Figure 21:

Cases 8, 20, 65, 55, 17 and 7



CASE NO.	DAYS AFTER ONSET	TITRE IgG	IgA	IgM
14	9	40	160	80
	23	320	320	320
15	Pre	<20	<20	<20
	5	80	20	40
	38	1280	160	320
	280	640	160	80
16	Pre	<20	<20	<20
	139	<20	20	40
	433	40	20	20
	554	80	<20	40
48	12	80	320	160
	34	640	160	160
	182	160	20	40

Table 13. Multiple samples from confirmed cases.

Figure 22.
Case 14

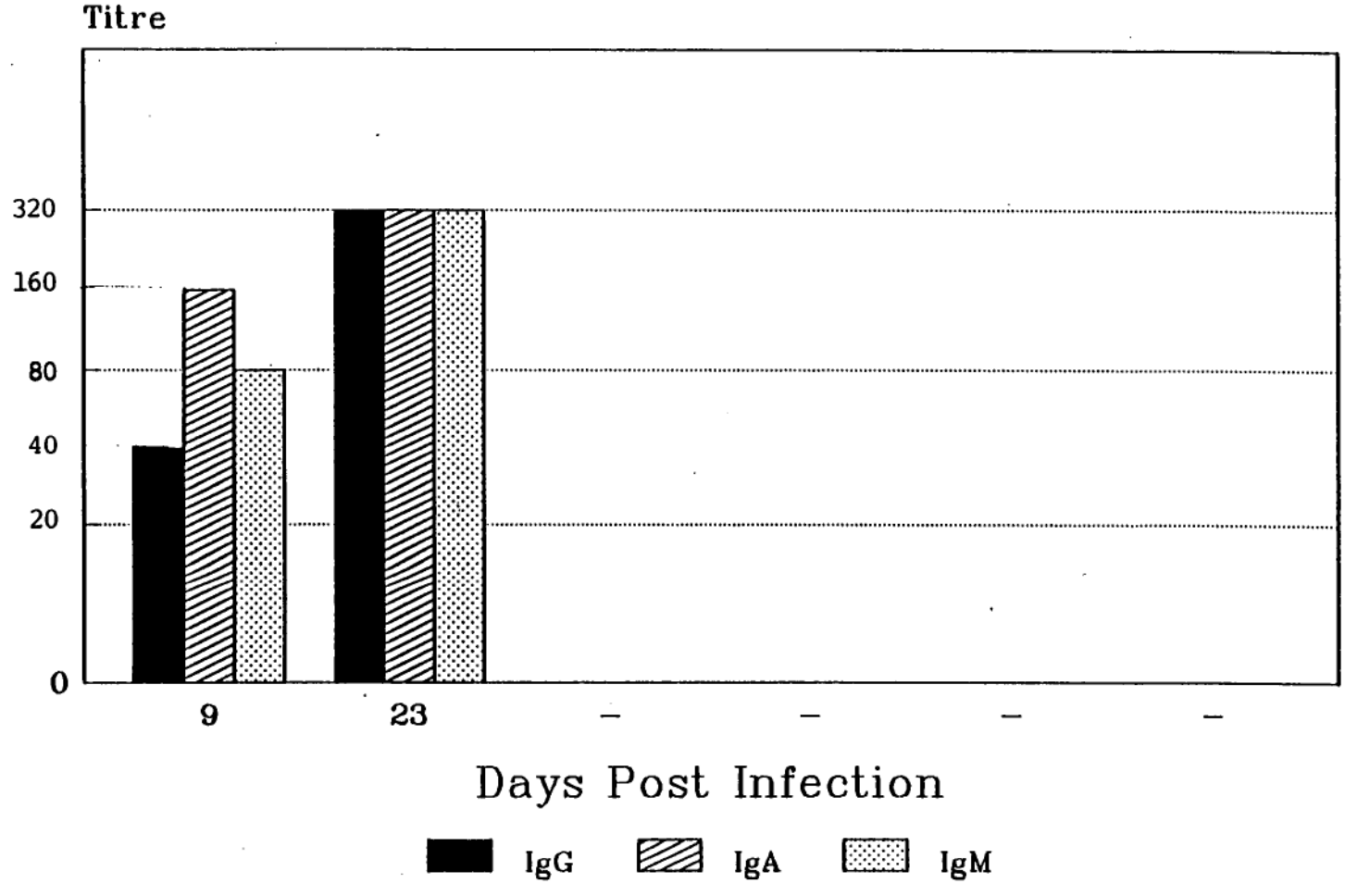


Figure 23.
Case 15

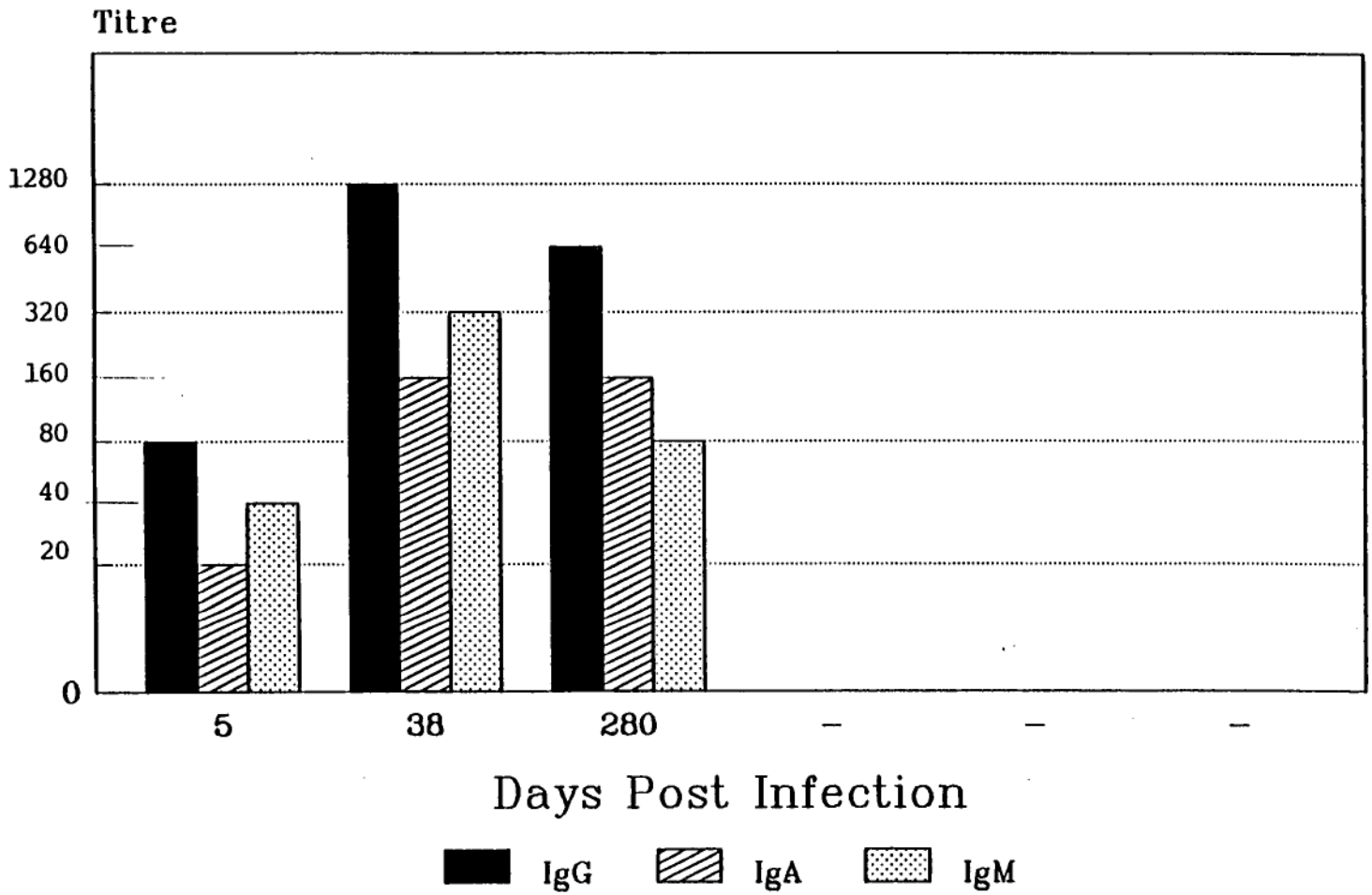


Figure 24.

Case 16

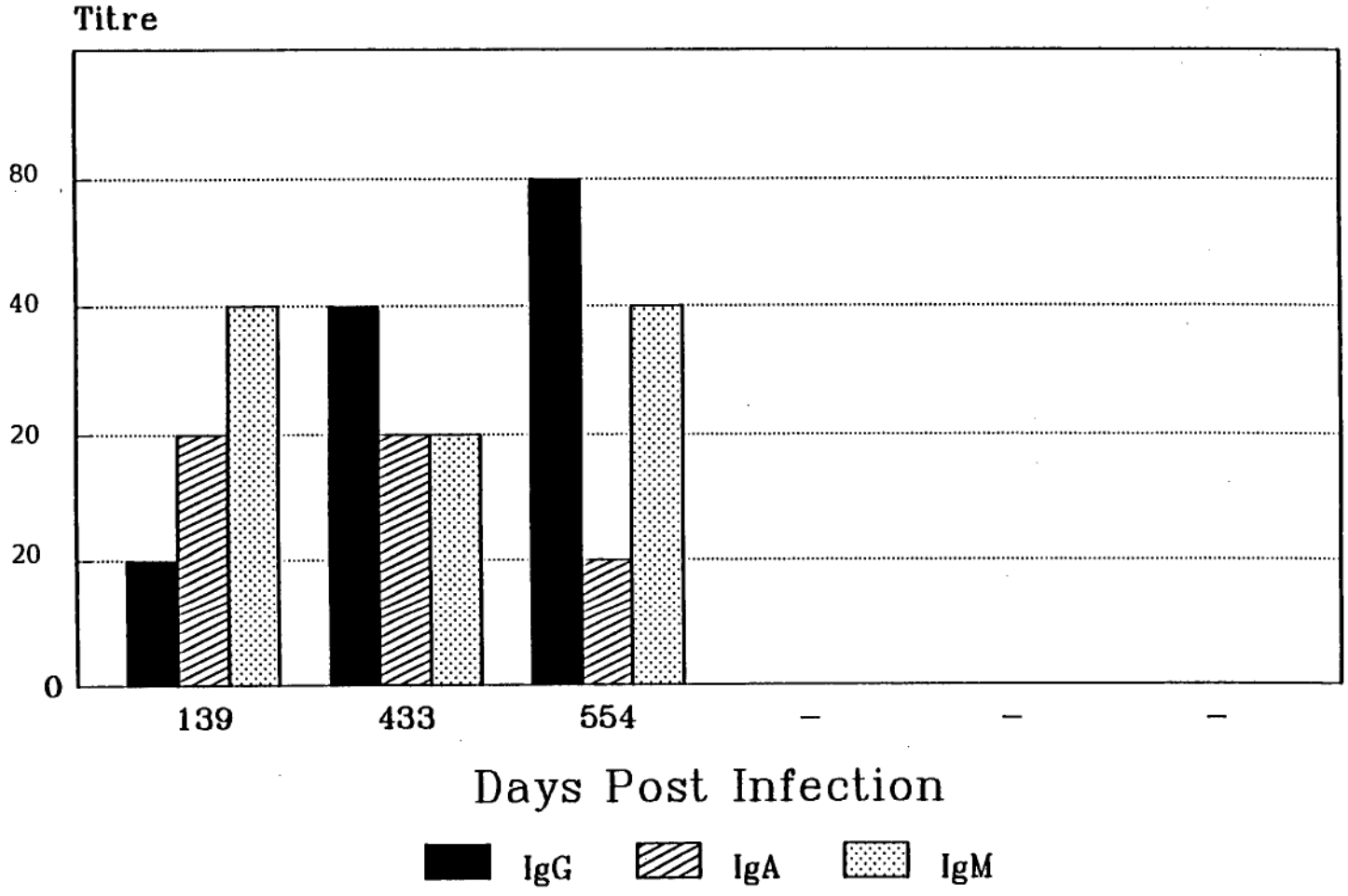
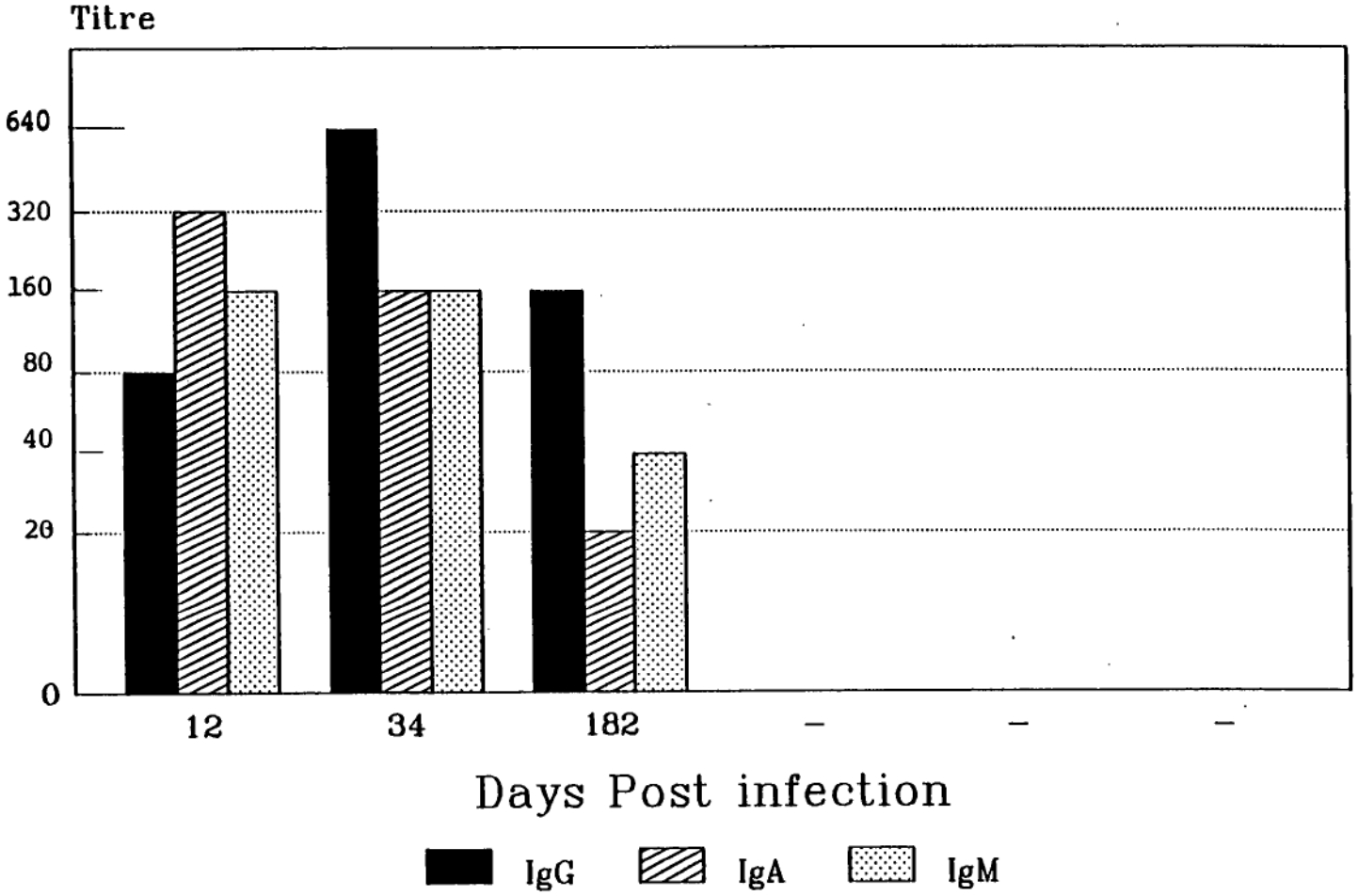


Figure 25.
Case 48



The two cases for which there were pre-infection samples available for testing and the two patients with positive Toxoplasmosis serology showed no antibodies to *Cryptosporidium* present. Case 16 may have become reinfected with *Cryptosporidium* between days 139 and 544 as the levels of IgG and IgM rose slightly. The patient was a practising homosexual who had biannual checks for HIV antibodies and occasionally suffered from bouts of diarrhoea. Case 52 was a 10 year old male haemophiliac with Group IV, Sub-group C1 (previously Catagory A) AIDS who showed little or no humoral immune response to cryptosporidiosis.

5.3 DISCUSSION

An immune response is known to arise from infection of the gastro-intestinal tract with many agents, some of which may be superficially located. Even though *Cryptosporidium* develops a 'pseudoexternal' location, an immune response has been previously shown (Ungar et al, 1986; Casemore, 1987b). The sporozoites and merozoites are initially extracellular, motile and capable of active invasion. Active cellular self-destruction, known to take place in enterocytes, may result in antigen being presented to the immune system.

The results of this study show that there appears to be an early humoral immune IgA response to cryptosporidiosis followed by IgM and then later IgG. All patients with confirmed cryptosporidiosis demonstrated an immune response of some kind. Nearly all samples obtained early in the infection showed a raised IgA level whereas samples obtained later showed an elevated IgG but little IgA or IgM. Patients with 2 or more samples available generally demonstrated a rise of IgA initially with IgG rising and remaining elevated and IgM rising and then declining along with the IgA. Case 15, a young woman in early pregnancy, was the most noticeable exception due to a remarkable early IgG response and comparatively low levels of IgA. There is no readily accessible explanation for this

happening. Pre-infection samples showed no evidence of previous exposure to *Cryptosporidium*.

The random control group study of urban and rural subjects, not known to have been previously exposed to or infected by *Cryptosporidium*, was performed to show that the organism is more prevalent in the community than has been previously shown by identification of the disease by demonstration of oocysts in faecal samples. No attempt was made to statistically analyse the results of the control group study due to the small size of the control population

In all but one case, IgG antibodies were the only class identified in the urban and rural control groups. The one control that demonstrated the presence of IgA and IgM also, was from an urban environment. The control group showed no clear preponderance of antibodies in either the urban or rural sectors, nor any great difference in the number of control patients with antibodies from the different parts of Tasmania, although the Launceston area showed the highest percentage followed by Hobart and then the North-west coast.

A larger control group would probably have revealed that the incidence of antibodies to *Cryptosporidium* is higher in the Launceston area, as this is the area from where the largest number of cases have been found. The

presence of antibodies in control patients from Hobart and the North-west coast shows that the organism is almost certainly present in these communities and that for some reason is not being identified.

As early as 1981, Tzipori described the presence of antibodies to *Cryptosporidium* in humans and animals. Then Campbell and Current (1983) demonstrated a human immune response in normal and immunodeficient patients with confirmed infections. They also found no cross-reactivity in their tests with other coccidia. None of these authors defined precisely the nature of the immnue response in terms of class of antibodies. Ungar et al, in 1986, studied the IgG and IgM response to *Cryptosporidium* in patients and controls including a large number of AIDS sufferers. Casemore (1987b) studied the immune response to all classes of antibodies and found a poor IgG response in early infection and that the IgA and IgM classes rose and then declined slowly. The rural controls in his study showed the presence of IgG antibodies while the urban controls often showed IgA and IgM antibodies. His findings relate well to the findings of this study. The level of antibody titres in Casemore's study (Table 4, page 45) were somewhat lower than those in this study which may reflect the relative insensitivity of the method he used. The possibility of re-infection having occurred in case 16 has been noted previously (Campbell and Current, 1983; Casemore, 1987b)

and suggests that protective immunity may not necessarily be conferred. The patients lifestyle could contribute to the increased number of gastrointestinal complaints that he suffered and a second undiagnosed bout of cryptosporidiosis cannot be excluded.

The one patient with Group IV, Subgroup C1 AIDS showed very low levels of IgG and IgA antibodies and no detectable IgM antibodies 42 days after the onset of symptoms. He died 28 days later. The study by Campbell and Current (1983) showed that patients with AIDS and persistent diarrhoea due to *Cryptosporidium* showed high titres of antibodies (unclassed). Ungar et al (1986) found that patients with AIDS usually demonstrated IgG antibodies even when their underlying disease was end stage but that IgM was not present later in the illness. This may be due to the fact that the IgM had already disappeared or that they were incapable of IgM response. No study on the presence of IgA santibodies in patients with AIDS has thus forth been recorded.

6 CONCLUSION

Although one staining method alone may not be completely effective in the diagnosis of cryptosporidiosis, the rapid auramine/carbol-fuchsin stain combined with faecal parasite concentration was found to be simple and sensitive and could be routinely performed in any diagnostic laboratory with minimal effort and expense.

The 30 month survey shows that *Cryptosporidium* is an important "new" pathogen in Tasmania. With increased awareness by microbiologists the organism has been shown to be present in many cases of gastrointestinal disease in this state. It is still apparent that many cases go undiagnosed due to lack of effort and awareness in some laboratories.

Earlier surveys of giardiasis and other parasites have been performed in Tasmania, and the results of these show an incidence of giardiasis comparative, at the time, to those of cryptosporidiosis now. A study by Goldsmid (1980) showed the prevalence of *Giardia lamblia* to be 3.3%. A further study involving children aged 0-5 years old revealed a 2.8% infection rate (Goldsmid et al, 1984). Outbreaks of giardiasis have also been reported in Tasmanian day-care centres and in families (Goldsmid et al, 1984).

As well as *Giardia lamblia*, a significant number of

cases of imported parasites such as *Strongyloides* (1), Hookworm (4), *Entamoeba histolytica* (2), and *Trichuris trichiura* (1) were identified during the survey period, mainly from migrant South-east Asians. Interestingly, the single case of *Ascaris lumbricoides* (identified by passing an adult worm) had never travelled out of Tasmania.

Surveys of imported parasites into Tasmania have been recorded in the past. Goldsmid, (1979), reported 20 cases of exotic parasites during an 18 month period, including *Trichuris trichiura*, hookworm, and *Entamoeba* species.

Demonstration of human immune response to *Cryptosporidium* is an breakthrough in the diagnosis of cryptosporidiosis. Detection of *Cryptosporidium* specific IgA antibodies probably indicates recent infection or contact and raised levels IgG antibodies probably suggests past infection. It's use in the diagnosis of cryptosporidiosis in patients with AIDS has also been shown to be valuable. Much more immunological research work needs to be done on known cases and patients with AIDS to prove this hypothesis.

The only other documented study of this magnitude on human immune response to *Cryptosporidium* was performed by Casemore in Great Britain in 1987. His findings were similar to those of this study and mostly support the

evidence in relation to the class of antibody found in known cases at varying times post infection and class of antibody found in urban and rural control groups.

A preliminary study was performed to detect the presence of coproantibodies to *Cryptosporidium* using the method described by Mahajan et al (1972) with modifications to suit the IFA test. The results of this trial were unsatisfactory and suggest the need for a better technique to be devised. This was unfortunate as some authors (Tzipori et al, 1986; Casemore, 1987b) suggest that *Cryptosporidium* may stimulate secretory IgA or IgG and the detection of these antibodies in faeces could, theoretically, be of diagnostic significance.

High costs of reagents and equipment has meant that this unfunded research project has to be suspended temporarily, although collection of suitable bloods for immunological studies in the future, and the surveillance and epidemiological studies of all faecal pathogens will continue.

The role of *Cryptosporidium* in human disease is now firmly established, emphasizing that it is an important pathogen even in the fully immunocompetent patient. This first study confirms that this is true in Tasmania also. In view of the increasing incidence of AIDS and the lack of a

satisfactory chemotherapeutic agent, it is important that surveillance work, immunological studies, and therapeutic research on this parasite should continue.

7 APPENDIX

7.1 STAIN EVALUATION METHODS

CARBOL FUCHSIN NEGATIVE STAIN

1. Mix equal quantities of fresh or formalin fixed or concentrated faeces and strong carbol fuchsin on a microscope slide and smear into a thin film.
2. Air dry.
3. Add immersion oil and cover with a coverslip.
   Examine with x40 brightfield objective.

Everything except the oocysts stain dark pink while the oocysts are bright and refractile because they contain water. The preparation must be examined within 10 to 15 minutes, before the oocysts collapse.

GIEMSA STAIN

1. Make thin faeces smears and air dry.
2. Fix in methanol for at least 10 minutes.
3. Stain with freshly diluted Geimsa stain (Koch-Light 1:30) for 1 hour.
4. Rinse gently with tap water and air dry.
   Examine using high power and oil immersion.

Oocysts stain pale blue and may have red inclusions visible in the inner matrix.

AURAMINE / PHENOL-CARBOL FUCHSIN

1. Thick faecal smears are air dried.
2. Stain for 5 minutes with auramine phenol
3. Rinse with tap water.
4. Countersain with strong carbol fuchsin 5 to 10 seconds.
5. Rinse and dry.

   Examine using fluorescent microscopy and low magnification.

Oocysts appear as very characteristic brightly fluorescent discs against a dark red background. Positive smears may be re-examined at high power and oil immersion.

SAFRININ-METHYLENE BLUE STAIN

1. Smear the sample, diluted in saline if necessary, on a microscope slide to a thickness slightly greater than necessary for routine bacteriological examination.
2. Air dry.
3. Fix briefly, by one pass through the bunsen flame.
4. Fix in 3% HCl in 100% methanol, 3-5 minutes.
5. Wash with water.
6. Stain with 1% aqueous safranin, 60 seconds.

   Heat thoroughly, preferably until boiling occurs.

   Add more stain and continue heating if necessary.

7. Wash with water.
8. Counterstain with 1% methylene blue, 30 seconds.
9. Wash with water, blot dry.
10. Fix coverslip with suitable mountant, e.g. D.P.X. (B.D.H.).

Examine using x20 objective.

Oocysts are seen as vivid orange-pink bodies approximately 5 m diam., usually spherical or slightly ovoid.

MODIFIED ACID-FAST

1. Make thin faecal smears and place on 70°C heating block for 10 minutes.
2. Stain with carbol fuchsin.
3. Gently heat to steaming (DO NOT BOIL).
4. Allow to react for 5 minutes.
5. Rinse with tap water.
6. Decolourise with 5% H_2SO_4 solution for 30 seconds.
7. Rinse with tap water.
8. Counterstain with methylene blue for 1 minute.
9. Rinse with tap water and air dry.

Examine using high power and oil immersion.

Oocysts stain deep red to pink with the residual body sometimes visible.

KINYOUN ACID-FAST

1. Make thin faecal smears and air dry.
2. Fix in methanol for 5 minutes.
3. Stain with Kinyoun carbol fuchsin for 15 to 20 minutes.
4. Wash well with tap water.
5. Decolourise with 7% H_2SO_4 for 60 seconds.
6. Wash well with tap water.
7. Counterstain with 5% malachite green.
8. Rinse with tap water and air dry.

   Examine using high power and oil immersion.

Oocysts stain deep red to pink with the residual body sometimes visible.

7.2 SURVEY METHODS

CONCENTRATION WITH THE FPC SYSTEM

1. Place a spoonful of faeces into 9ml of formal saline
   in the flat-bottomed tube provided.
2. Add 3 drops of 20% Triton X-100 and mix well.
3. Fit the conical tube including the filtration unit to the flat-bottomed tube and invert.
4. Shake the contents through the filter until transferred to the conical tube.
5. Add 3 ml of ethyl acetate and shake well.
6. Centrifuge at 2000rpm for 2-4 minutes.
7. Decant supernatant after carefully releasing ethyl-acetate/faecal debris plug.
8. Prepare iodine and sucrose float preps and make a smear for staining for *Cryptosporidium*.

AURAMINE / PHENOL-CARBOL FUCHSIN

1. Thick faecal smears are air dried.
2. Stain for 5 minutes with auramine phenol
3. Rinse with tap water.
4. Counterstain with strong carbol fuchsin 5 to 10 seconds.
5. Rinse and dry.

   Examine using fluorescent microscopy and low

magnification.

Oocysts appear as very characteristic brightly fluorescent discs against a dark red background. Positive smears may be re-examined at high power and oil immersion.

KINYOUN ACID-FAST

1. Make thin faecal smears and air dry.
2. Fix in methanol for 5 minutes.
3. Stain with Kinyoun carbol fuchsin for 15 to 20 minutes.
4. Wash well with tap water.
5. Decolourise with 7% H_2SO_4 for 60 seconds.
6. Wash well with tap water.
7. Counterstain with 5% malachite green.
8. Rinse with tap water and air dry.

   Examine using high power and oil immersion.

Oocysts stain deep red to pink with the residual body sometimes visible.

7.3 IMMUNE RESPONSE METHODS

PREPARATION OF ANTIGEN

Percoll

Percoll (Pharmacia) is a substance consisting of colloidal silica particles that have been coated with polyvinylpyrrolidone (PVP) which can form gradients in the range 1.0-1.3 g/ml, and is completely non-toxic to cells. Its low viscosity facilitates very rapid isopycnic banding or rate zonal separation of cells or particles.

a. Making a working solution of Percoll

Percoll may be diluted to lower densities by diluting with 0.15 M saline for cell work or with 0.25 M sucrose when working with sub-cellular particles or viruses. In a measuring cylinder add 1/10 of the desired final volume 1.5M NaCl or 2.5M sucrose (e.g. 10ml for 100ml of working solution). To this add the required volume of Percoll. The following formula can be used to calculate the volumes of Percoll required to obtain the desired density:

$$V_c = V \frac{p - 0.1p_{1c} - 0.9}{p_c - 1}$$

where V_c = volume of Percoll ml

V = volume of the final working solution ml

p = desired density of the final solution g/ml

p_c = density of Percoll (from bottle) g/ml

p_{1c} = density of 1.5 M NaCl = 1.058 g/ml

For the experiment, working solutions of Percoll of densities 1.04g/ml, 1.08g/ml, 1.017g/ml and 1.03g/ml were needed. The calculations needed to produce 100ml of each follows:

$$\text{Volume of Percoll} = 100x\frac{1.08 - 0.106 - 0.9}{0.13}$$

= 57.0ml to produce 1.08g/ml in 0.15M NaCL of Percoll

add to 10ml of 1.5M NaCl and make up to 100ml with distilled water.

$$\text{Volume of Percoll} = 100x\frac{1.04 - 0.106 - 0.9}{0.13}$$

= 26.1ml to produce 1.04g/ml in 0.15M NaCl of Percoll

add to 10ml of 1.5M NaCl and make up to 100ml with distilled water.

$$\text{Volume of Percoll} = 100x\frac{1.03 - 0.106 - 0.9}{0.13}$$

= 20.0ml to produce 1.03g/ml in 0.15M NaCl of Percoll

add to 10ml of 1.5M NaCl and make up to 100ml with distilled water.

$$\text{Volume of Percoll} = 100x\frac{1.017 - 0.106 - 0.9}{0.13}$$

= 8.4ml to produce 1.017g/ml in 0.15M NaCl of Percoll

add to 10ml of 1.5M NaCl and make up to 100ml with distilled water.

The method described by Casemore, suggested the use of 20% Percoll, which calculated back using the formula:-

$$V_c = V\frac{p-0.1p_{1c}-0.9}{p_c-1}$$

where V_c = volume of Percoll ml

V = volume of the final working solution ml

p = desired density of the final solution g/ml

p_c = density of Percoll (from bottle) g/ml

p_{1c}= density of 1.5 M NaCl = 1.058 g/ml

gives a Percoll solution of density approximately 1.03g/ml although he found it to have a density of 1.017g/ml using the Pharmacia Density Marker Beads.

The method described by Waldman et al (1986) uses Percoll at concentrations, 1.04g/ml and 1.08g/ml.

b. Analysing the density gradient of Percoll

To check the density gradient of Percoll and to standardise running conditions before carrying out an actual experiment, it is possible to generate a series of gradient curves specific for a particular centrifuge rotor using the Pharmacia Density Marker Beads. There are 10 types of beads, each colour coded and modified to have a specified density in gradients of Percoll. Vials of beads

are reconstituted by adding 1ml of distilled water giving approximately 0.6ml when swollen in aqueous medium. Buoyant density of the different beads is shown in Table 14.

Vial No	Colour	Buoyant density (g/ml) in Percoll	
		0.15 M NaCl	0.25 M Sucrose
1	Blue	1.016	1.032
2	Orange	1.033	1.049
3	Green	1.048	1.050
4	Red	1.062	1.070
5	Blue	1.077	-
6	Orange	1.087	1.099
7	Green	1.100	1.112
8	Red	1.118	1.133
9	Violet	1.139	1.135
10	Blue	-	1.085

Table 14. Buoyant density values of Pharmacia Density Marker Beads.

The beads are then used as external markers in a centrifuge tube containing identical gradient material to the one used in the experiment. The beads are added to the control tube in 10-15ul aliquots which is then used as the counter-balance in the rotor during centrifugation.

c. Preparing a density gradient of Percoll

For the first experiment, 3ml of the 1.04g/ml Percoll was carefully layered on to an equal volume of the 1.08g/ml

Percoll in 10ml conical glass centrifuge tubes by using a syringe fitted with an 18-guage needle. The material containing the *Cryptosporidium* oocysts was then layered on top of the Percoll. In the second experiment, the Percoll was used in two lower concentrations of 1.03g/ml with 1.017 g/ml over and the oocyst containing material layered on top.

d. Centrifugation of the density gradient of Percoll

Centrifuge rotor geometry, the time and speed of centrifugation and the size of the tubes used, are important in determining the shape of the gradient of Percoll formed. The centifuge used in the experiment was a Digifuge (Foss Electric) with sealed swing-head rotor. The speed and time of centrifugation were determined by experiment using predetermined guidelines in the booklet "Percoll: methodology and applications". A speed of 3000rpm for 10 minutes appeared to provide an adequate separation of material in the gradients. After centrifugation the bands of material in the gradients were examined microscopically for the presence of *Cryptosporidium* oocysts. The oocyst rich bands were evaluated in a counting chamber as to the density of oocysts present and then washed once in PBS pH 7.3 to remove the Percoll. The purity of the deposit was then examined for contamination.

Sucrose

Sucrose is useful in the separation *Cryptosporidium* oocysts from faecal material. A modified method of that developed by Heyman et al, 1986, was used to purify *Cryptosporidium* oocysts from faeces.

a. Preparation of sucrose solutions

An initial sucrose solution was prepared by diluting 454g sucrose in 355ml of distilled water. This was then diluted to yield 10, 20, 30 & 50% solutions for use in density centrifugation.

b. Preparation of sucrose density gradient

Starting with the 50% solution, 10ml of each successive dilution was transferred dropwise with a Pasteur pipette into a 50ml screw-cap centrifuge tube. A 5ml sample of the material to be separated was then layered onto the sucrose gradient.

c. Centrifugation of sucrose density gradient

The tube was centrifuged at room temperature for 10 minutes at 1000 x g to obtain pellets at the 30% and 20% levels. The two pellets were then washed once with saline to remove the excess sucrose and quantitated microscopically for oocyst concentration and purity.

ANTIBODY DETECTION TECHNIQUE

The method used for detection of *Cryptosporidium* antibodies in serum was an indirect immunofluorescent technique. Fluorscein iso-thio-cyanate (FITC)/antiglobulin conjugates used were sheep anti-human globulin (AHG), anti-IgG, anti IgM and anti-IgA (Wellcome, Australia)

Before finalising the methodology it was necessary to experiment to establish the best way of running the assay. Three major factors needed to be decided and these were as follows:-

1. Dilution factor of the of the FITC conjugated anti-human immunoglobulins.
2. Time and temperature of the incubation steps.
3. Appearance of oocysts microscopically at the cut off point in the titration.

The first two problem factors were solved in a two fold experiment using different dilutions of the FITC conjugated anti-human immunoglobulins and variable incubation times and temperatures. Dilutions of the AHG conjugate (Wellcome, Australia) was made from 1 in 20 to 1 in 160. A dilution of 1 in 80 was found to give satisfactory results. For the anti-IgA and IgM (Wellcome) a suitable working dilution was 1:10, and for the anti IgG (Wellcome) a working dilution of 1:20 was found to give

satisfactory results. After many trials, incubation times of 30 minutes at 37°C were found to give satisfactory results to the test.

The third problem was solved during the multiple tests on the dilution and incubation factors. Negative sera gave no fluorescence around the oocysts, whereas positive sera gave a weak to strong fluorescence around the oocysts.

Preparation of slides

Purified *Cryptosporidium* oocysts were diluted in saline to give a concentration of five to ten oocysts per high power field. For use the antigen was spotted on Teflon-coated, multispot microscope slides (Wellcome Australia). Minute amounts of antigen only were applied to each well and air dried. The slide preparations were then fixed in acetone for 5 minutes and stored at 4°C ready for use.

Preparation of serum samples

Doubling dilutions of serum from 1 in 5 up to 1 in 10420, depending on the clinical status of the patient, were prepared in small test tubes using PBS pH 7.3 as the diluent. Aliquots (25ul) of each dilution of serum was added to coded wells on the prepared slides. These slides were then incubated at 37°c for 30 minutes.

The slides were then carefully rinsed with PBS pH 7.3 using a wide nozzle squeeze bottle, then washed for 10 minutes with PBS pH 7.3 in a slide jar, rinsed with distilled water and allowed to drain for 1 minute.

Aliquotes (25ul) of the appropriate, suitably diluted fluoscein iso-thio-cyanate (FITC)/anti-globulin conjugate were added to the wells and the slides incubated as before. At the end of the second stage of incubation, slides were rinsed as before and then washed in PBS pH 7.3 containing 1% Evans Blue 'counterstain' for 10 minutes, rinsed with distilled water and then mounted in PBS-glycerol with a coverslip.

The slides were examined with an Olympus Fluorescence Vertical Illuminator microscope at x40 dry.

7.4 CASE STUDIES

Clinical histories and information on possible routes of infection were not available on every patient and therefore all the patients will not have case studies recorded here. Those that do, represent a cross-section of the more interesting cases as well as those that may be related to a known source.

Case 2

A twenty seven year old woman from a non urban settlement presented to her local doctor with diarrhoea, abdominal pain and some vomiting for 5 days. *Cryptosporidium* oocysts were found in her stools which were liquid and foul-smelling, and she was approached for further samples. At that stage, 9 days after onset, she had lost 7 Kg in weight and was not passing faeces. At 11 days she produced a formed stool which she reluctantly sent in to the laboratory complaining that it still smelt revolting but that she felt considerably better. Scanty oocysts were still present in the second sample. The patient had no contact with animals and said she rarely left the house.

Case 5

A 22 month old boy from a small east coast township suffered persistent diarrhoea for two weeks before stool samples were referred to the laboratory. He was generally

well otherwise. Scanty oocysts were seen in the concentrated preparation at which stage he was admitted to hospital with severe cramps, diarrhoea and vomiting. Large numbers of oocysts were then found in his stools at this later stage.

Case 6

A nine year old boy from the same non-urban community as case two was diagnosed 3 months later as having cryptosporidiosis. He first presented with abdominal pains and by the time the scanty cryptosporidia were seen in the stained preparation from the concentration, he had developed profuse diarrhoea, vomiting and severe abdominal pains. Further stools were examined and found to contain large numbers of oocysts. The patient's family property adjoined farmland with sheep being the only livestock farmed. The family also drank unpasteurised milk, as did many other people in the community.

Case 7

A four year old boy, resident in Launceston with a history of various abdominal episodes for about four months. The most recent bout had commenced three weeks prior to the faecal sample being submitted to the laboratory for examination. The major symptom at the time was persistent vomiting. Scanty oocysts were seen in the sample and a diagnosis of cryptosporidiosis made although

no conclusions as to the role of *Cryptosporidium* in the child's earlier symptoms could be drawn.

Case 8

A thirty one year old urban male was hospitalised with diarrhoea, vomiting, and severe dehydration. A faecal sample revealed numerous oocysts on day five of his illness and day two of the diarrhoea. Five days before the onset of the illness the patient had been to a farm and participated in the milking of the cows. He admitted to spending most of the time at the "wrong end" of the cow. Two days after being admitted to hospital and receiving intensive supportive therapy he had considerably improved.

Case 9

A six year old girl with a three day history of diarrhoea and abdominal pain. Numerous oocysts were detected in her foul-smelling liquid stools. Five days prior to becoming unwell she had visited a farm and played all day with an orphaned lamb that had the "skitters". The lamb has since died. The diarrhoea was preceded by a period of three days of lassitude, anorexia and abdominal discomfort all of which continued during the illness.

Case 10

The mother of the six year old girl complained of abdominal pain and anorexia when presenting her daughter's

follow up faeces to the laboratory. The next day she developed diarrhoea and obliged with a specimen for examination. Moderate numbers of oocysts were seen in the sample and later, when interviewed, the mother said that she felt considerably better and that her daughter was "back to normal".

Case 11

A six year old boy living in an suburban area was hospitalised with vomiting and diarrhoea of eight days duration. Seven days prior to the onset of diarrhoea he had been on a school trip to a zoo and wild-life park. Three days after that he visited a farm and consumed fresh cow's milk. His mother said that other children on the school trip became unwell with diarrhoea and vomiting also. After his release from hospital he suffered from lassitude and anorexia for some days.

Case 13

A two year old girl from a rural property with "alternate lifestyle" parents was found to have *Cryptosporidium* oocysts in her stools. She was well cared for but suffered from recurrent diarrhoea. No previous faecal samples had been submitted and small numbers of oocysts were seen in a well formed specimen. Eleven months later her baby brother (case 31) was also diagnosed as having cryptosporidiosis.

Case 14

A forty eight year old city dwelling male had suffered from diarrhoea of five days duration when he presented to his doctor. Three days later a faecal sample was delivered to the laboratory and small numbers of *Cryptosporidium* oocysts were seen. He had visited a property that adjoined a goat farm about 5 days before the onset of his illness where he petted some dogs that played in the goat paddock. He also often consumed unpasteurised milk as did his wife and she admitted some anorexia and lethargy during the same period as her husband although no diarrhoea was evident. The patient volunteered to provide a post infection serum sample for antibody studies.

Case 15

In the same non-urban community as cases two and six a 24-year old pregnant woman was diagnosed as having cryptosporidiosis after she suffered diarrhoea, vomiting, nausea, lassitude and anorexia. A serum sample was obtained prior to her illness, at the time of her illness and also at the time of her confinement 8 months later and stored for antibody studies. It may be interesting to note that unpasteurised milk is sold at the local shop and delivered by the milkman in this community.

Case 16

A thirty one year old male homosexual, known to be promiscuous, presented with symptoms of prolonged diarrhoea. *Cryptosporidium* oocysts were found in his stools. Follow up samples at 5 weeks post infection (to exclude AIDS) and at 6 months (during another bout of diarrhoea) showed no *Cryptosporidium* present. As a regular attender at the laboratory for screening for HIV antibodies, serum samples from pre and post infection were available for testing for cryptosporidial antibodies.

Case 17

A 6 1/2 year old boy residing on a dairy farm presented with diarrhoea and was diagnosed as having cryptosporidiosis. His doctor complied with a request for a post infection blood sample and also responded with a sample from the child's mother who had not suffered any symptoms but had consumed the same food as the child. Results of serological tests for cryptosporidiosis on the mother showed evidence of recent exposure to *Cryptosporidium*.

Case 20

A 29 year old man with a history of 3 days diarrhoea and abdominal pains was diagnosed to have cryptosporidial enteritis. He was known to have consumed unpasteurised milk recently. As he had also suffered from recurrent sore

throats, malaise, loss of weight, and night sweats, a follow up request was made for HIV antibody studies. The blood received was also saved for cryptosporidial antibody studies.

Case 22

A 49 year old woman was admitted to hospital with severe abdominal pain not unlike appendicitis or mesenteric adenitis. While on the theatre list for an emergency laporotomy, the pain subsided and she developed fever and mild diarrhoea. A stool sample revealed large numbers of *Cryptosporidium* oocysts and her name was removed from the theatre list. The patient admitted to being a lover of raw milk and made special trips to a certain dairy farm to obtain her nirvana. Further investigation showed the aforementioned farm to be in the midst of calving at the time of her last sortie, thus making unpasteurised milk the prime suspect for her unfortunate situation.

Case 27

A 7 year old boy who had recently spent the school holidays on his divorced fathers farm with his 3 siblings developed severe diarrhoea due to *Cryptosporidium*. While on the farm he consumed raw milk as did his siblings who also suffered a mild diarrhoea. After diagnosis was made his mother complained of diarrhoea, as did her boyfriend, highlighting the possibility of person to person spread

although no stool samples were available from the rest of the family. This case also highlights the difference in intensity of infection, as none of the other children became sick enough for proper investigation.

Case 36

A 26 year old man was diagnosed as having cryptosporidiosis after spending 3 weeks working as a contract carpenter at a rural abattoir. He had no other history of possible exposure to the organism.

Case 45

A 16 year old girl became unwell at asthma camp, where 10 out of the 70 children there also had a 24hr bout of gastro. The girls illness persisted for 2 weeks and at that point a diagnosis of cryptosporidiosis was made. As the girl lived on a "hobby farm" with goats, sheep, and cattle, the source of the cryptosporidial infection was unclear.

Case 48

A 26 year old man suffered diarrhoea and vomiting for 12 days before being referred for faecal analysis. A diagnosis of cryptosporidiosis was made and the patient volunteered for further blood samples to be taken. He complained of feeling run down and lethargic for many weeks after the initial bout of illness and had been more prone than usual to colds and 'flu'.

Case 52

A 10 year old haemophiliac boy, who had received a transfusion of HIV contaminated Factor VIII resulting in development of antibodies to HIV, suffered chronic diarrhoea of six weeks duration before being diagnosed as having cryptosporidiosis. As the chronic diarrhoea was assumed to be due to *Cryptosporidium*, this placed the previously non-AIDS patient into Group IV, Subgroup C1 (previously Catagory A) AIDS. The patient had been treated with AZT since the detection of HIV antibodies two years previously. After the diagnosis of cryptosporidiosis he had received a course of hyperimmune cow milk and spiromycin with no evidence of improvement. The patient died 4 weeks later from unremitting diarrhoea.

Case 55

A 41 year old woman was hospitalised with severe gastroenteritis. Large numbers of *Cryptosporidium* oocysts were found in her faeces. She remained unwell for a considerable time after her initial illness and some serum samples were available for antibody studies.

Case 66

A 34 year old diabetic man was diagnosed as having cryptosporidiosis after suffering prolonged diarrhoea, including abdominal pain, anorexia, lethagia and nausea. He resided in a situation where the community milked their own cows to supply the people with milk (unpastuerised) and several other people had similar ailments at the time but less severe. He complained of extreme "rumbling" in his abdomen, which may have been due to the fermentation process.

Case 78

A 74 year old lady with persistent diarrhoea was diagnosed as having cryptosporidiosis after blaming the "meals on wheels" for being the cause of it. No evidence of this being the case has so far come to light.

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